MECHANISMS FOR THE CLEARANCE OF BLOOD IN THE VITREOUS

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PREFACE

Hippocrates[214] stated the general law that "blood which spills into a cavity must decay". From his writings, it is clear that he was referring to blood in the peritoneal or pleural cavities and to the subsequent inflammatory response that such deposits elicited. Since his work predates the invention of the ophthalmoscope by some twenty-two centuries, he probably had little knowledge of haemorrhage into the vitreous cavity. Had he been aware of this condition, he might have regarded it an exception to his rule.

Blood may clear rapidly and completely from the vitreous cavity with full restoration of vision, provided the retina is intact. Conversely, the vitreous may remain opaque for weeks, months or even permanently in the most serious cases, resulting in blindness. The relative increase in blindness due to bleeding into the vitreous humour can be attributed to the prevalence of diabetic retinopathy with which vitreous haemorrhage is closely associated. Moreoever, the prognosis for sight is unpredictable in this condition.

Aside from the obvious serious visual effects of vitreous opacities due to blood, the failure of blood to clear from the vitreous is intriguing, since it implies a failure of the normal scavenging mechanisms of the body in removing unwanted tissue debris. The studies described in this thesis were initiated by this apparent defect in the normal healing process and by the lack of knowledge generally on the fate of blood in the vitreous. Previous studies of human material have shown that persistent vitreous opacities contain suspensions of degenerate red cells plus dense collagenous vitreous bands or "membranes". Experimental studies have also demonstrated that several processes such as haemolysis, phagocytosis and fibroblastic activity occur during the reabsorption of vitreous clots in rabbits. Despite this body of work, a cause (or

causes) for the delayed resolution of vitreous clots has not been identified.

Studies of the interaction between blood and tissues involve several physiological systems including coagulation, fibrinolysis, haemolysis, the inflammatory response and the wound healing response, which are all closely interrelated. The present work is an experimental study which was designed to examine a number of these systems in relation to the interaction between blood and vitreous.

Plan of Thesis

The thesis is divided into four parts. In Part 1, the clinical background of vitreous haemorrhage is outlined and a brief description of the normal vitreous body is included. Part 2 comprises an investigation of the mechanism of blood coagulation in the vitreous. Part 3 contains experimental data which delineates the role of fibrinolysis in the resolution of vitreous blood clots. In Part 4, a study of the sequential pathological changes occurring within experimental vitreous clots is described and an attempt is made to resolve the controversy concerning the source and formation of vitreous "membranes". A summary of the findings is reported on page XXXI and a short appendix is included which sets this experimental work into the context of therapy of human vitreous haemorrhage (Chapter 15).

SUMMARY

This thesis explores the pathophysiological mechanisms involved in the clearance of vitreous blood clots from the eye. The work presented is an experimental study of various aspects of the blood-vitreous interaction, including coagulation and fibrinolytic mechanisms in the vitreous, and the morphological changes which occur during lysis of experimental vitreous blood clots in vivo.

Initial investigations were aimed at establishing the mechanism of blood coagulation in the vitreous. The effects of vitreous and its components on humoral and cellular coagulation mechanisms were studied. The presence of humoral procoagulant activity in normal vitreous was sought using standard cell-free plasma-derived coagulation assays. Where necessary, purified coagulation factors were prepared and used to identify specific coagulation factor activities in the vitreous. It was found that the vitreous contained minimal tissue thromboplastin. In addition, trace amounts of a Hageman factor-like activity were detected in normal vitreous both by clotting studies and by activation of pre-kallikrein. No other coagulation factor activities were detected. Pure hyaluronic acid, which is essentially the sole glycosaminoglycan of vitreous, had little effect on the intrinsic system blood clotting mechanism when tested with activated plasma. However, both hyaluronic acid and vitreous induced a significant shortening of the recalcification time of non-activated plasma. A role for hyaluronic acid in the activation of Hageman factor was suggested by these results.

The role of platelets in fibrin formation within the vitreous was also investigated. Both rabbit and bovine vitreous collagens caused rapid platelet aggregation in spite of their unusual nature. However, evidence for fibrin formation as a direct result of platelet activation and in the absence of other mechanisms, could not be established. It was postulated therefore that intravitreal clotting occurs in vivo through

activation of blood-derived Hageman factor by the vitreous, or more specifically, by its glycosaminoglycan component, the process being amplified by rapid platelet aggregation. Tissue thromboplastin may contribute to the reaction in a minor fashion. It is possible, therefore, that coagulation in the vitreous is initiated via the intrinsic clotting pathway, differing from clotting in other injured tissues where release of tissue thromboplastin is considered of major importance.

The second phase of the study consisted of an investigation of the role of fibrinolysis in clearing vitreous blood clots. Fibrinolytic activity, as well as components of the plasminogen-plasmin system, were assayed in normal vitreous using established techniques. Plasminogen activator activity in the vitreous from all species tested was found to be minimal (equivalent to 15 Ploug units of urokinase per ml of vitreous). Accordingly, an experimental model of vitreous haemorrhage was developed to assess the role of fibrinolysis within resolving vitreous clots. A standard volume (0.2 ml) of autologous whole blood was injected into the rabbit eye, and the opacification of the vitreous, as seen by ophthalmoscopy, was correlated with the level of intravitreal fibrinolysis. Fibrinolysis was estimated by measuring the concentration of fibrin and fibrin degradation products (FDP) within vitreous clots after various time intervals. It was shown that fibrin remained within the vitreous for at least five weeks and that FDP generation was low (5-20 μg/ml). Thus, although fibrinolysis occurred within the vitreous, the level of activity compared poorly with that in other tissues. In a further experiment, using the same animal model, in which intrinsic vitreous fibrinolytic activity was blocked by the synthetic inhibitor, tranexamic acid, fibrin clearance from the vitreous was only slightly delayed (six weeks), even though FDP generation was reduced to undetect-

able levels. Consequently, it was suggested that the poor resolution of vitreous haemorrhages in general may be related to the low levels of fibrinolytic activity in the vitreous, either directly by retarding the clearance of fibrin deposits, or indirectly, by failing to provide an adequate chemotactic stimulus through such agents as FDP or plasmin-mediated complement fragments, both of which are known to be chemotactic in vitro.

These experiments were extended to a study of the pathology of vitreous haemorrhage. The rabbit model described above was used, and the sequential changes in morphology were studied by macroscopic, histological and ultrastructural methods. Macroscopically, the vitreous clot remained as a discrete mass for four to six weeks, after which time it became considerably reduced in size. During this period, it changed colour from dark red to brown and eventually became a pale white mass. The presence of blood exerted severe destructive effects on the gel structure of the vitreous, including posterior detachment of the solid vitreous, liquefaction of the gel, the production of prominent vitreous bands or "membranes" and the formation of a pseudocapsule by the detached vitreous around the blood clot. Small blood deposits persisted in several eyes for weeks or months.

Detailed study of the inflammatory response to intravitreal blood revealed a low-grade cellular reaction with a notable lack of polymorphonuclear cells. Mononuclear cells formed large aggregates and multinucleate giant cell formation was evident. These cells were actively engaged in removing cellular debris mainly derived from degenerate red cells, which had commenced autolysis at an early stage. It is possible that this extracellular haemolysis was initiated by the relative anoxia and low glucose concentration within the vitreous, and sustained in the later stages by macrophage enzyme secretion. Furthermore, fibroblastic activity was not observed in any of the specimens examined. The ultra-

structural evidence suggested that the so-called "fibrous" bands or "membranes" of clinical description resulted from co-agmentation of vitreous collagen strands.

The conclusion drawn from this study is that persistence of blood clots in the vitreous is the consequence of a number of factors probably peculiar to the vitreous. The low levels of fibrinolytic activity, and the postulated absence of an adequate chemotactic stimulus, may have been partly responsible for the low-grade, atypical inflammatory response. In addition, the vitreous itself may have contributed to the poor cellular response, since it contains a considerable quantity of high molecular weight hyaluronic acid, a substance known to inhibit inflammatory cell function in vitro. These observations are not only pertinent to vitreous pathophysiology, but since hyaluronic acid is a ubiquitous macromolecule, they may have some relevance to the general field of inflammatory mechanisms.

Finally, dense bands or "membranes" can develop after bleeding into the vitreous, without the presence of fibroblast activity. It is suggested that fibrous organisation is an uncommon sequel to uncomplicated vitreous haemorrhage, and is found in ocular conditions where vitreous bleeding may be an incidental occurrence.

PART I

INTRODUCTORY

CHAPTER 1

THE CLINICAL PROBLEM OF VITREOUS HAEMORRHAGE

INTRODUCTION

Bleeding from intraocular vessels into the vitreous cavity has long been recognised as a cause of blindness[287]. Moreover, the unpredictable clinical pattern of vitreous haemorrhage reabsorption was well known to the early ophthalmoscopists[201,215,356]. Lawson[284] in his treatise on 'Diseases and Injuries of the Eye', observed that visual loss due to bleeding into the vitreous was often permanent since blood clots in the vitreous gel often failed to clear spontaneously. Regrettably, the clinical picture of untreated vitreous haemorrhages remains the same today[31,270,295,342].

There are many factors which affect the prognosis of vitreous haemorrhage resolution, some of which are related to the special nature of the vitreous body. A brief description of the composition of the normal vitreous body is therefore included in this chapter.

THE NORMAL VITREOUS

In most mammals, the entire space between the retina and the lens is filled with a transparent solid gel known as the vitreous body (Fig.1). In the normal eye of a young adult, the vitreous volume is about 4 ml, i.e. somewhat more than two thirds of the intraocular volume. With age and/or development, a certain portion of the gel becomes liquid, mainly in the central part. A few species have an entirely liquid vitreous such as the owl monkey (douroucouli Aotus trivirgatus)[26] and the bush baby (Galago crassicaudatus agisymbanus)[299]. Although the vitreous is 99% water and only 1% solids, it is regarded as a true tissue[183], since it is composed of the same constituents as other tissues, namely cells, fibres and interstitial ground substance[21]. A

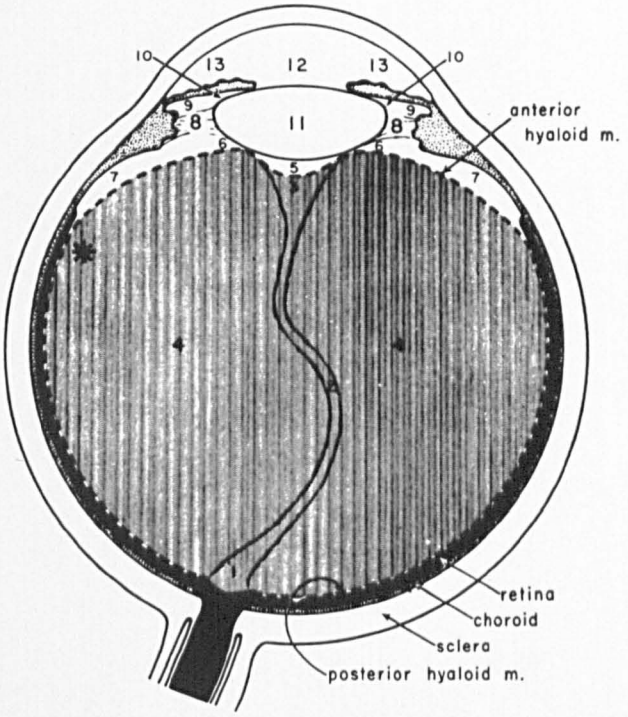


Figure 1.1 Schematic diagram of adult human eye showing the topographical anatomy of the vitreous. Areas or "zones" are numbered after Cibis[88]. Zones 1-4 (shaded areas) represent the vitreous. 1, prepapillary space of Cloquet's canal; 2, intermediate space of Cloquet's canal; 3, retrolental space of Cloquet's canal; 4, space around Cloquet's canal. Zones 6-11 represent the posterior chamber. 8, zonule; 11, lens. Zones 12-13 represent the anterior chamber. Berger's space is a potential space (zone 5) between zones 3 and 11. Zone 7 represents Petit's canal. * area of vitreous base. The cortical vitreous is a 1 mm thick outer layer of the vitreous gel bounded by the posterior hyaloid membrane and in close apposition to the retina.

major difference, however, between vitreous and other tissues is its avascularity.

Gel formation in the vitreous is due to the fibrous protein, collagen[26,197,315,400] which in the human occurs at a concentration of about 0.3 mg/ml[21]. The distribution of collagen, however, within the vitreous is not uniform; liquid vitreous contains no collagen, while the highest concentration is found near the vitreous base, less so in the cortical vitreous (Fig. 1.1). More recent studies of vitreous collagen suggest that different types of collagen may exist in different species (for review of collagen types, see reference 334). Bovine vitreous collagen appears to be Type II[492], while rabbit vitreous collagen resembles Type IV and appears to be associated with lipid material[492]. Studies of vitreous collagen formation in the chick embryo using immunofluorescent techniques also show the presence of Type II collagen, although study of Type IV collagen was not possible due to lack of appropriate antibody[525]. However, the possibility that vitreous collagen may represent a separate genetically distinct type of collagen has also been raised[358] It has been suggested that vitreous collagen has a dual origin from neural retina and hyalocytes[358] (vide infra).

Electron microscopic studies of vitreous collagen also lay stress on its unique nature. Vitreous structure is formed by delicate collagen fibrils of a width somewhere between 60A° and 250A° and a periodicity between 120° and 300°A[139,174,464]. The collagen fibrils are anchored in a basal lamina which lines the retina of the adult eye and is firmly attached to the cell membranes of the retinal Muller fibres. Detachment of the vitreous fibres from the retinal surface leads to rupture of the Muller cell membrane and produces a "posterior hyaloid membrane" composed of condensed vitreous collagen fibrils, basal lamina and Muller cell debris[23,146]. The basal lamina is absent at the vitreous base, and here the collagen fibrils are

directly adjacent to the epithelial cells[427]. The presence of a distinct anterior hyaloid membrane separating the vitreous from the posterior chamber has long been debated, but electron microscopic studies have failed to reveal such a structure. Instead, a concentration of vitreous collagen fibrils forms the posterior wall of the posterior chamber[23].

The ground substance of most tissues contains a variety of glycosaminoglycans[396]. However, the interstitial matrix of the vitreous contains predominantly hyaluronic acid, with only traces of sulphated glycosaminoglycans[491]. The concentration of hyaluronic acid in the vitreous shows considerable interspecies variation, low levels being found in the rabbit (0.03 mg/ml) and high values in the owl monkey (0.5-0.8 mg/ml)[21]. Human vitreous hyaluronic acid concentration varies with age between 0.1 and 0.5 mg/ml. Hyaluronic acid is also distributed unevently in the vitreous, highest levels being found in the posterior cortical layer next to the retina, and decreasing towards the centre and anteriorly[491]. The molecular weight of the hyaluronic acid molecule, which in cattle varies between 6×10^4 and 3.5×10^5 daltons, increases in the reverse direction[21]. Morphological identification of hyaluronic acid is extremely difficult since most of the material is lost in the fixation and dehydrating processes used for preparing tissues for microscopy. Some authors have suggested that hyaluronic acid molecules can be detected with ruthenium red staining on electron microscopy[465]. Hyaluronic acid imparts viscosity to the vitreous and may also assist in maintaining the stability of the three-dimensional collagen network[22]. It may also act as a molecular sieve for the passage of molecules between the vitreous and other ocular structures[280].

The third macromolecular constituent of the vitreous is soluble protein. Cooper et al[107] found a vitreous protein concentration in the

range of 0.4 to 0.8 mg/ml and detected a wide variety of serum proteins, plus some that were immunologically distinct vitreous tissue antigens. Although serum proteins are present in the vitreous, their concentration ratios do not correspond to those in the serum, and this suggests that they have not arrived in the vitreous merely by ultrafiltration[27,45]. The distribution of soluble proteins was observed to vary according to the locality[27], but this may have been an artefact associated with the freezing technique used[491].

The cellular content of the vitreous is low, about one million cells per cattle vitreous[28] (volume 8-9 ml). They occur as a monolayer in the cortical tissue layer of the vitreous, and electron microscopic studies have indicated that they may have a phagocytic function[56,157] since they contain numerous lysosomes. However, it is clear that vitreous cells, or hyalocytes, as they have been described[21], contain the necessary enzymes to elaborate hyaluronic acid and it is possible that this is their major function[377]. In addition, they may be involved in collagen production[358]. At present, in spite of their having been identified over 130 years ago (cf reference 200) their primary role remains obscure.

The topographical anatomy of the vitreous body is shown in figure 1.1. The various structures and spaces are identified by number. Cloquet's canal is best seen in vivo, using the biomicroscopic method of slit lamp examination. It is composed of concentrically arranged condensations of vitreous collagen that form a sheath, known also as the plicate membrane. Numerous other channels and folds have been identified within the vitreous from time to time, but these are probably artefactual and have little proven physiological significance.

CAUSES OF VITREOUS HAEMORRHAGE

Haemorrhage into the vitreous usually originates from retinal vessels, less frequently from choroidal or ciliary body vessels. It follows that diseases of the retinal vessels are likely to be associated with vitreous haemorrhages. This association has been recognised since the early days of ophthalmoscopy, and indeed retinal neovascularisation, associated with disorders such as retinal vein occlusion or diabetes, is a well-established cause of vitreous haemorrhage[311,502]. Furthermore, the clinical entity of recurrent vitreous haemorrhages in young men with inflammatory disease of the retinal vessels was first described in 1882 by Eales[128]. A causal relationship with tuberculosis or syphilitic disease was suggested for this condition. Vitreous haemorrhage in association with retinal hole formation and retinal detachment has also been well recognised, but the size of the bleed is usually very small. Occasionally, however, large intragel haemorrhages have been observed[524]. Similarly, an association between generalised bleeding disorders and vitreous haemorrhage has been noted[190].

Several reports have described the clinico-pathological features of vitreous haemorrhage[90,255,319], and case reports of intravitreal bleeding in a wide variety of systemic and local disorders have been recorded on numerous occasions. However, systematic study of the incidence and frequency of causes of vitreous haemorrhages is relatively recent. Jaffe[236] classified the causes of vitreous haemorrhage into nine categories: (1) increased retinal venous pressure (e.g. central retinal vein occlusion, sickle-cell disease, subarachnoic haemorrhage, polycythemia vera), (2) increased retinal arterial pressure (e.g. hypertension), (3) inflammation of the retinal veins (e.g. in Eales' disease, or secondary to uveitis), (4) retinal arteriovenous malformations (e.g.

Coats disease, Leber's miliary aneurysms, retinal angiomas), (5) retinal neovascularisation (e.g. diabetes mellitus, central and branch venous occlusion, Eales' disease, sickle-cell disease, retrolental fibroplasia), (6) retinal tears or hole formation (usually secondary to vitreous detachment), (7) following cataract extraction (from anterior chamber, retina or choroid), (8) bleeding disorders (e.g. disseminated intravascular coagulation, idiopathic thrombocytopenia, and various anaemias and dysproteinaemias (e.g. Waldenstrom's macroglobulinaemia) and (9), tumours (e.g. malignant melanoma, retinoblastoma). Other causes are trauma (either blunt or penetrating injuries) and congenital or developmental disorders of the vitreous such as persistent hyperplastic vitreous and congenital retinoschisis.

INCIDENCE OF VITREOUS HAEMORRHAGE

The incidence of vitreous haemorrhage in the general population is difficult to determine, since by present diagnostic classifications such cases are usually recorded under the causal disease, e.g. diabetic retinopathy, or retinal vasculitis. Recent studies[31,295,342] suggest that diabetic retinopathy is the most common cause of vitreous haemorrhage at present, and in one series[342], this disorder accounted for more than fifty per cent of two hundred consecutive cases. In a separate study of 161 eyes with diabetic retinopathy, the incidence of vitreous haemorrhage was found to be 72 per cent[512]. Since the incidence of diabetes in the general population is estimated at 1.5 per cent, and the prevalence of diabetic retinopathy among diabetics about 50 per cent[109] it is possible that the incidence of vitreous haemorrhage in the general population may be as high as 0.54%. This figure is almost certainly an overestimation, due to selected data sampling. A more

realistic figure can be derived from the incidence of proliferative retinopathy in the diabetic population, which ranges between 4.43 to 20.0 per cent[109,442,560]. If it can be assumed that vitreous haemorrhage in diabetics occurs mainly in relation to neovascularisation, and that seventy per cent of such patients are likely to have some degree of vitreous haemorrhage[512], an incidence of 0.05 to 0.35 per cent of the population can be expected for vitreous haemorrhage due to diabetes alone. Statistical estimates of this nature, however, are notoriously inaccurate. What is more certain is that blindness due to diabetes has increased considerably. In 1940, 4.3% of blindness was caused by diabetes, while by 1962 the figure had increased to 18.4%[357]. Much of this visual disability is associated with vitreous haemorrhage. Indeed, Caird[72] has estimated that 31% of diabetic eyes are registered blind one year after the onset of vitreous bleeding. In view of the increased life expectancy of diabetics and the known association of retinopathy with the duration of diabetes, it can be expected that the incidence of vitreous haemorrhage will increase pari passu.

In the absence of diabetes, the most frequent cause of vitreous haemorrhage is retinal tear[295,446]. Other common causes of vitreous haemorrhage include hypertension and retinal venous occlusion[31]. The remaining disorders associated with vitreous haemorrhage[236] are much less frequent. In addition, although retinal haemorrhages are commonly seen in the neonatal period[85], vitreous haemorrhages are much rarer[251], and the possibility of a coagulation defect should be considered in such circumstances[566].

CLINICAL FACTORS ASSOCIATED WITH DELAYED RESOLUTION OF VITREOUS HAEMORRHAGES

Several clinical factors may alter the prognosis for vision in cases of vitreous haemorrhage. These include the size, locality, cause, and recurrence rate of the haemorrhage, and the presence of complications. Large haemorrhages resolve less readily than small extravasations of blood[31,295], and haemorrhages into the vitreous gel also resolve much more slowly than those into the subhyaloid (or retrogel) space. It has been suggested that the rapid reabsorption of blood from the subhyaloid space is due to the absence of clotting in this situation and thus the red cells sediment to the lower part of the eye where they are rapidly reabsorbed[531]. Fluid levels within subhyaloid haemorrhages have long been recognised[314] and this strongly supports the idea of a failure of blood clotting in the subhyaloid space. However, no experimental data has been produced to support this concept. Moreover, it is possible that subhyaloid haemorrhages produce less disability because they are of smaller size than intragel haemorrhages and therefore are more readily absorbed. Haemorrhages may also occur in other localities within the vitreous, such as Cloquet's canal[32] or Petit's canal, or between the lens and the anterior hyaloid face (Berger's space)[256] (see Fig.1.1).

The cause of a vitreous haemorrhage may also influence its reabsorption. Diabetic vitreous haemorrhages have a poorer visual prognosis than non-diabetic clots, but this may be related to the presence of complications such as neovascular or fibrous tissue in the vitreous, rather than to the clot itself. However, insulin-dependent diabetics have also been reported to have reduced fibrinolytic activity in their plasma[137] and this may affect the reabsorption of the blood from the vitreous (see summary).

Finally, it is self-evident that recurrent vitreous haemorrhages, such as occur in Eale's disease or diabetic retinopathy, have a poorer visual prognosis than single bleeds.

DIAGNOSIS OF VITREOUS HAEMORRHAGE

The diagnosis of vitreous haemorrhage is usually simple. A sudden diminution of vision is accompanied by the presence of visible opacities in the vitreous cavity on ophthalmoscopy. A small haemorrhage may produce only a few small discrete opacities which can be seen to move in conjunction with ocular movements. By contrast, a large haemorrhage will completely obscure visible fundal details. A clot of this nature may persist indefinitely, but if spontaneous reabsorption occurs, visible strands can be detected in the centre of the vitreous, often followed in the later stages by the redistribution of the partially resorbed blood clot to the lower part of the globe. In this situation, it is common to see white or grey-white masses in the lower globe, interspersed with black or even bright-red clot material. Further reabsorption may lead to the development of "vitreous membranes", a term used to describe the appearance of the dense bands which traverse the vitreous cavity and which may obscure vision. Slit-lamp examination of vitreous blood reveals a remarkable picture of dense "vitreous veils" in which cellular material can be identified even into the late stages of reabsorption.

Diagnostic problems arise in attempting to distinguish between simple vitreous haemorrhage and vitreous haemorrhage associated with other ocular disease, e.g. diabetic retinopathy, retinal detachment or tumour. In such cases, the history and the examination of the fellow eye are often of great assistance, but frequently the vitreous opacities preclude a definitive diagnosis. The recent application of ultrasono-

graphy to ocular diagnosis has considerably improved the evaluation of eyes with vitreous haemorrhage, since typical echo patterns from various intraocular lesions can be obtained[97,98,150,373].

COMPLICATIONS OF VITREOUS HAEMORRHAGE

"Spontaneous" vitreous haemorrhages, as described by Morse et al[342] to distinguish them from haemorrhages induced by ocular trauma, produce no pain and little or no inflammation in the eye. Cases have been observed in which no change occurred in the blood clot for several months or years, and then rapid and complete resolution occurred over a period of a few weeks with full visual recovery[29]. However, most cases of vitreous haemorrhage reabsorb slowly, if at all, and it is widely believed that non-resorbing blood clots can be complicated principally by two conditions which considerably worsen the visual prognosis. The first is the occurrence of fibrosis within the blood clot, which eventually leads to contraction of the vitreous, and, in areas of firm vitreo-retinal adhesion, retinal hole formation and retinal detachment. Cases of this nature have been observed most frequently in eyes with retinal neovascularisation from diabetes or other retinal vascular disease, or in eyes which have suffered severe trauma (for discussion of the pathogenesis of vitreal fibrosis in vitreous haemorrhage, see Chapter 10). It is therefore unclear whether the fibrotic reaction results from the presence of the blood in the vitreous or from the original disorder, or both. Duke-Elder[126] hints at this problem in his description of post-haemorrhagic retinitis proliferans, in which he states that not only is the presence of blood in the vitreous required to stimulate a fibroblastic reaction, but "a sufficient degree of irritation, neither too much nor too little", is also necessary.

The second complication which may follow vitreous haemorrhage is glaucoma. Two forms of open angle glaucoma have been described, haemolytic and haemosiderotic. Haemolytic glaucoma is caused by large inflammatory cells, red cells and haemolysed debris (erythroclasts) passing from the vitreous to the aqueous during absorption of the vitreous haemorrhage and blocking the aqueous outflow channels[163,230]. Haemosiderotic glaucoma is caused by direct toxicity of iron on the trabecular meshwork[90,519], while the angle of the anterior chamber remains open. The diagnosis of these conditions is a pathological one, and the incidence is unknown.

Retinal damage after vitreous haemorrhage has also been described mainly on pathological[17,90,564] or experimental[90,369,422] grounds. However, the recovery of visual function following spontaneous or therapeutic clearance of vitreous blood would suggest that the incidence of such retinal damage is not high, at least to a clinically detectable degree.

THERAPEUTIC CONSIDERATIONS

Since this thesis is aimed at an understanding of the basic pathophysiological mechanisms in vitreous haemorrhage reabsorption, the treatment of vitreous blood clots is not considered at this stage. However, a brief summary of current trends in therapy is included in Chapter 15.

PART 2

COAGULATION MECHANISMS WITHIN THE VITREOUS

CHAPTER 2

REVIEW OF PHYSIOLOGY OF BLOOD COAGULATION

INTRODUCTION

The classical theory of blood coagulation proposed by Morawitz[338] in 1905 and based on a concept of four interacting factors, was considerably altered by the enzyme cascade theory of Macfarlane[321] and Davie and Ratnoff[115]. The latter theory was introduced to account for the increase in knowledge concerning blood coagulation factors which at that time numbered at least twelve. According to the cascade theory, several factors present in blood combined in an orderly sequence to convert soluble fibrinogen to fibrin (Figure 2.1). Surface activation of Factor XII initiated activation of Factors XI and IX which in turn led to activated Factor VIII and produced activated Factor X in the presence of phospholipid and ionic calcium. Thrombin was then generated via activated Factor V and finally fibrinogen was converted to fibrin.

However, clotting also occurs in the tissues and Blomback[53] proposed a similar scheme which separated coagulation into three phases, and included an extrinsic (tissue thromboplastin) mechanism in the second phase. According to this scheme, blood clotting in the tissues occurred via the extrinsic pathway in which Factor X was activated by the interaction of blood factor VII and tissue thromboplastin (T-Tbp) in the presence of calcium ions (Figure 2.2). A final common pathway for both intrinsic and extrinsic mechanism then occurred via Factor V and thrombin.

During the last decade, detailed biochemical investigations into the nature and interactions of individual clotting factors have suggested that this concept, although fundamentally correct, requires modification. For example, several factors such as Factor VIII and V are now considered to act not as enzymes but as regulatory proteins by enhancing the rate of conversion of their respective substrates to active factors[134]. In addition, the discovery of two asymptomatic disorders of clotting -

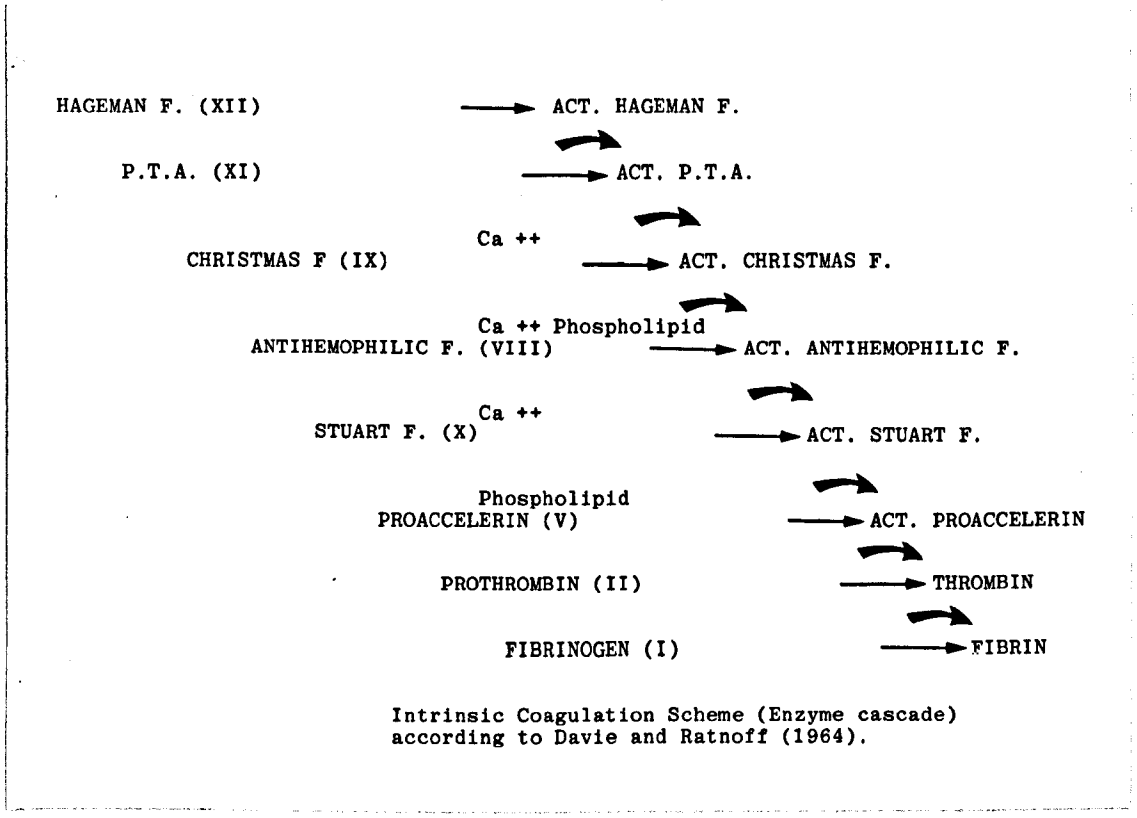


Figure 2.1 Intrinsic coagulation scheme (Enzyme cascade) according to Davie and Ratnoff[115]. It is now known that Factors VIII and V act as regulatory proteins or cofactors in the coagulation sequence and not as shown here (see text).

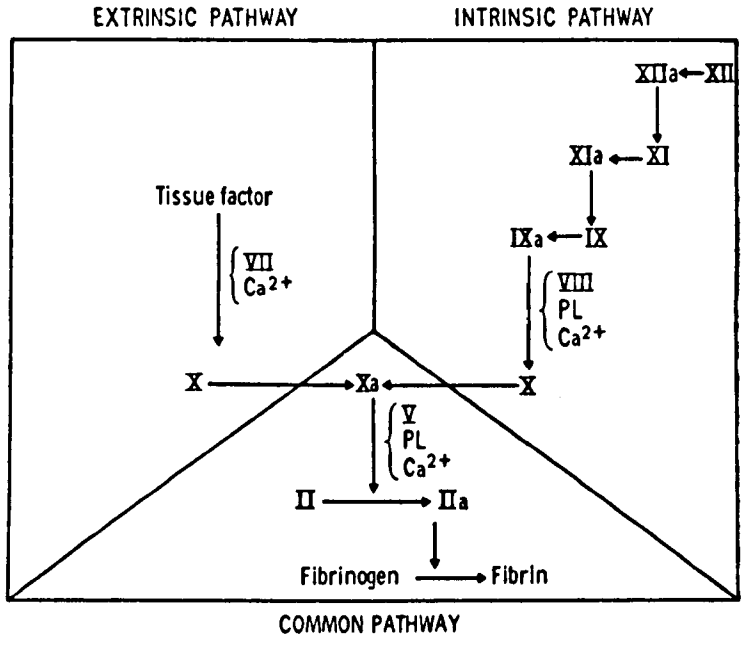


Figure 2.2 Pathways in blood coagulation (from Esnouf[134])

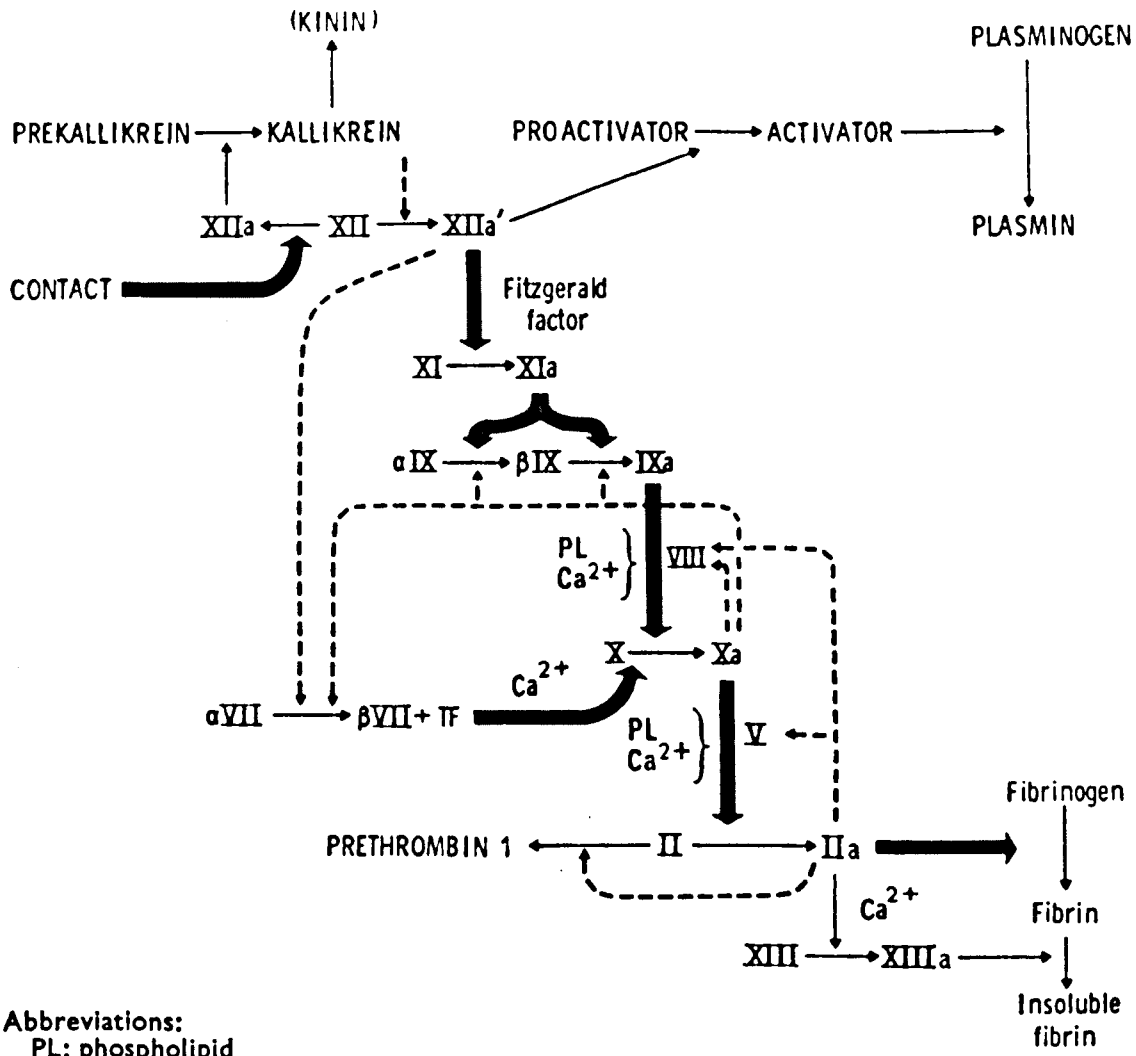
Fletcher trait[208] and Fitzgerald trait[532] - has provided further insight into the earliest stages of the intrinsic clotting pathway, and it is now generally accepted that both coagulation mechanisms interact in a highly integrated and balanced fashion with several other systems such as kinin formation and fibrinolysis. These pathways are discussed in more detail below.

INTRINSIC PATHWAY

Coagulation by the intrinsic pathway occurs via activation of Factor XII. Factor XII, or Hageman factor, has been purified by Schoenmakers et al[459,460] who considered it to be a sialoglycoprotein with esterolytic and proteolytic activity, and a molecular weight of 80,000[121]. Grammens et al[191], however, studied bovine Factor XII and produced a homogeneous preparation with a molecular weight of 140,000. Activation of Factor XII can be initiated by contact with a foreign surface, and it has been suggested that this is due to interaction between the high negative charge on the foreign surface (e.g. glass, talc, kaolin) and the positively charged amino-acid residues of the Hageman factor molecule[418]. More recent studies that utilised the technique of circular dichroism have shown that a conformational change occurs in the molecule on contact with a foreign surface[326] and this change probably confers marked hydrophobicity on the molecule. This activated moiety (XIIa) may then convert prekallikrein or Fletcher factor - a gammaglobulin proenzyme that circulates in the plasma complexed to high molecular weight kininogen (HMW, or Fitzgerald factor)[310] to kallikrein, which rapidly activates more Factor XII[95]. This second reaction, however, differs from contact or surface activation of Factor XII in that the molecule is cleaved to produce Fragment E (mol. weight

28,000)[428]. Fragment E is considered to be the active principle involved not only in the intrinsic coagulation scheme, but also in the activation of extrinsic system Factor VII and plasminogen (Fig. 2.3)[177]. However, recent studies have shown that cleavage of Factor XII during contact activation of plasma yields two fragments, an 80,000 mol. wt. fragment known as α-HFa and a smaller, 28,000 mol. wt. fragment designated β-HFa (Fragment E). α-HFa is a two chain enzyme capable of activating prekallikrein and factor XI in either surface - bound or fluid phase, while β-HFa is a single chain molecule which cleaves prekallikrein only[429].

In addition to the well-known activators of Factor XII such as glass, kaolin and talc, several agents of biological significance have also been reported as activators of Factor XII. These include collagen[203,559], vascular basement membrane[94], sebum[366] and bacterial lipopolysaccharides[341]. The negatively charged soluble molecules, ellagic acid[416] and chondroitin sulphate[345] are also known activators of Factor XII. The precise mechanism of surface activation of Factor XII is currently in debate, particularly regarding the role of Fitzgerald Factor or HMW kininogen. HMW weight kininogen is a glycoprotein with a molecular weight of about 76,000, and a preferential susceptibility to plasma kallikrein[259]. Digestion of single-chain HMW kininogen with kallikrein yields a kinin-free double chain molecule. Coagulant activity resides in the light chain of the molecule[504]. In addition to its role as "contact activation cofactor" in the generation of activated Factor XIa (see below), some workers believe that HMW kininogen is required for the conversion of prekallikrein and thus for Factor XII activation[82,457]. Moreover, high molecular weight kininogen may have a role in the activation of plasminogen via kallikrein[569]. This complex and unresolved subject has been reviewed recently by Ogston and Bennett[370]



Abbreviations:
PL: phospholipid
TF: tissue factor
XIIa': enzymically active fragment E

Thick arrows denote the action of enzymes on their specific substrates as envisaged in the cascade hypothesis. Positive and negative feedback loops are indicated by interrupted lines

Figure 2.3 Schematic representation of interaction between blood coagulation factors and the kallikrein and fibrinolytic systems (from Esnouf[134]).

and they have proposed a tentative scheme to outline the various interactions (Figure 2.4). Undoubtedly, this concept will change and may have done so already since the recent report of a plasma factor which increases Factor XII activation in the presence of HMW kininogen but is apparently distinct from Factors XII and XI, prekallikrein-kallikrein, HMW kininogen and plasminogen-plasmin[81]. The positive feedback system (Figs. 2.3 and 2.4) inherent in these processes ensures that surface activation of Factor XII, whatever its mechanism, is greatly enhanced once it has been initiated. Activated Factor XII then converts Factor XI to its active form in the presence of Fitzgerald factor, or HMW kininogen[453]. Little is known about the structure of Factor XI since it has been difficult to isolate, but its active form is a serine protease which initiates activation of Factor IX[250]. Factor IX is a glycoprotein with a molecular weight of 55,400 and it contains 26% of carbohydrate in association with a single polypeptide chain[162]. Activation of Factor IX involves two stages, firstly the cleavage of the protein chain to yield a light chain (mol. wt. 16,600) and a heavy chain (mol. wt. 38,000). Disulphide bonds connect the two split products[250]. The second stage is completed by the removal of a peptide from the N-terminal position of the heavy chain; this produces procoagulant activity in the molecule. Activated Factor IXa is a serine protease[114] which enzymically converts Factor X to Factor Xa and requires the presence of phospholipid, calcium ions and a further plasma protein, Factor VIII. In addition, it has been observed that incubation of Factor IX with a mixture of Factor Xa, phospholipid and calcium produces Factor IXa. Thus a possible link between the extrinsic and intrinsic pathways exists[241] (Fig. 2.3).

Factor VIII is a complex protein which has three properties: coagulant activity, Factor VIII-related antigen activity, and platelet aggregation activity in the presence of the antibiotic ristocetin. It

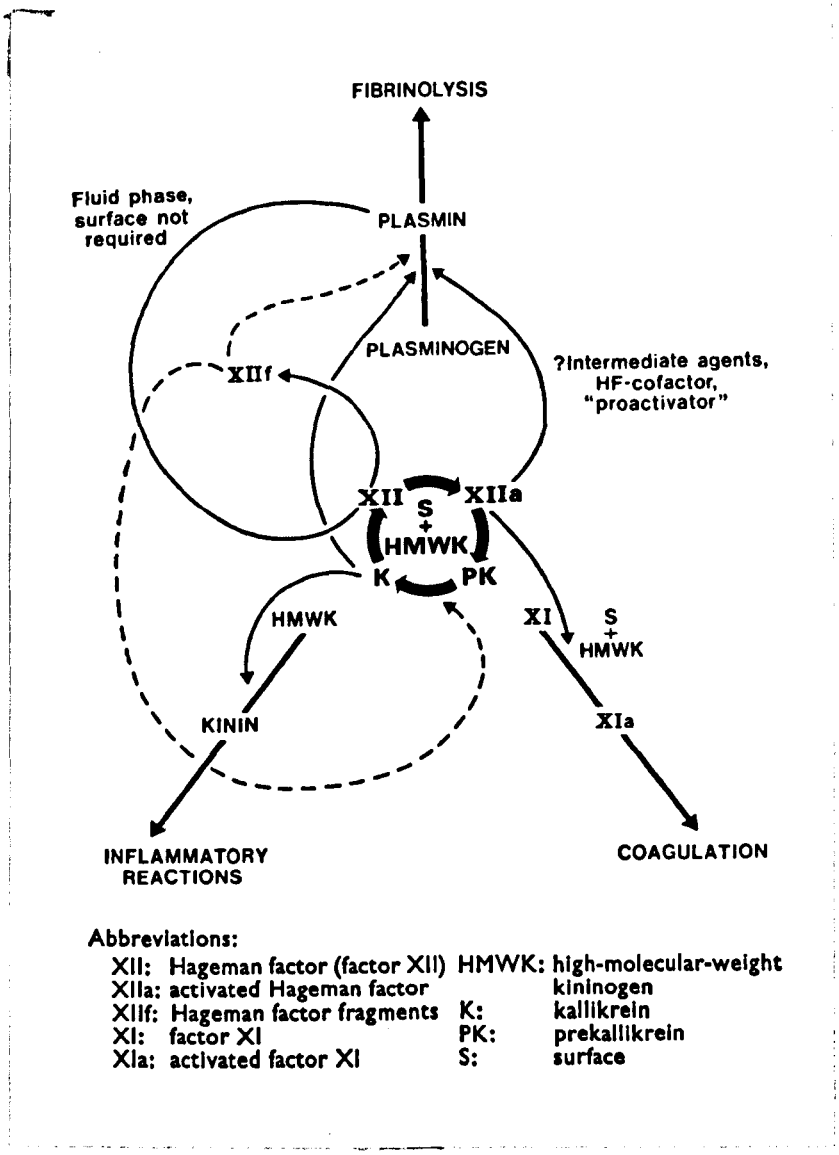


Figure 2.4 Hypothetical scheme for the central role of high molecular weight kininogen in fibrinolysis, coagulation and inflammation (from Ogston and Bennett[370]).

is not known whether these activities occur separately within the molecule, but it seems likely that they do, since each activity can be selectively inhibited by appropriate antibody[225]. Biochemical separation of these activities has only been partially achieved[66,494], but greater separation can be obtained using immunoadsorption techniques[266]. Preparations of Factor VIII from the cryo-precipitate fraction of plasma indicate that the molecular weight is about 1.2×10^6, and it is composed of glycoprotein subunits of m.w. $1\text{-}2 \times 10^5$, held together by disulphide bonds[269]. Current views on Factor VIII coagulant activity suggest that it acts as a regulatory protein in the conversion of Factor X to Factor Xa[114], and that Factor VIII requires traces of thrombin before it can act. Thrombin has been shown to catalyse the formation of a more active form of Factor VIII from both the purified molecule and the low molecular weight procoagulant fractions of Factor VIII[431].

EXTRINSIC PATHWAY

In the extrinsic pathway of coagulation as originally described, two factors interact, namely Factor VII from blood and tissue factor, or tissue thromboplastin, from the tissues. The reaction product converts Factor X to activated Factor X in the presence of calcium ions. Tissue thromboplastin is a lipoprotein complex which has widespread distribution in the organism[182]. When the lipid and protein components are dissociated, tissue thromboplastin loses its coagulant activity, but this can be restored when the protein is relipidated[487]. Although tissue thromboplastin was originally thought to be of lysosomal origin, it is now clear that it occurs mainly in the plasma membrane of cells, particularly vascular endothelium[574]. Recent studies, however, have shown that thromboplastic activity varies with cell type under tissue culture conditions.

It was shown that basal activity in endothelial cells was lower than for other cell types such as fibroblasts and they were also comparatively refractory to drug effects[318]. These findings were regarded as consistent with the proposed role for an intact endothelium in haemostasis. A further recent finding of interest is that the reaction product of Factor VII and tissue thromboplastin may activate Factor IX, thus demonstrating a second link between the extrinsic and intrinsic coagulation pathways[378].

Thromboplastic activity also varies with the tissue. High levels of activity were found in the lungs, brain and placenta[556,557] and also in several ocular tissues such as the retina and choroid[382]. The vitreous, however, has minimal procoagulant activity (see Chapter 4).

In addition to its interaction with Factor VII, tissue thromboplastin has considerable peptidase activity and it has been suggested that under normal circumstances the predominant role of tissue thromboplastin is the inactivation of small vasoactive peptides, while its procoagulant activity is masked, perhaps by a molecular sieve mechanism involving the glycocalyx[469].

Factor VII is a single chain glycoprotein (mol. wt. 45,500) which forms a complex with tissue thromboplastin before it converts Factor X to Factor Xa. Catalytic amounts of Factor Xa then convert Factor VII to a disulphide-bonded double chain which has 80 fold specific activity on interaction with tissue thromboplastin, and thus markedly augments the production of Factor Xa in a positive feedback mechanism[412]. In addition, this represents a third link between the extrinsic and intrinsic clotting pathways (Figure 2.3). Factor VII has a chemical composition very similar to other Vitamin K-dependent coagulation factors such as Factors II, IX and X, and besides being activated by Factor Xa, Factor VIIa can

be generated by kallikrein, plasmin, Hageman Factor fragment E, and Factor IXa. Furthermore, Factor VII_a is unaffected by known inhibitors of coagulation, such as antithrombin III and heparin (see later).

COMMON PATHWAY

Factor X is a glycoprotein with a mol. weight. of 55,000 and comprises two chains joined by a disulphide bridge[161]. It is activated by the intrinsic and extrinsic pathways, by insoluble trypsin and by Russel viper venom in a two stage process involving cleavage of identical peptide bonds[239]. Coagulant activity is found after the first stage. Factor Xa converts prothrombin to thrombin in the presence of Factor V, phospholipid and calcium ions. Factor Xa also catalyses the conversion of Factor VII (see above).

Factor V does not participate directly in the coagulation scheme, but greatly accelerates the production of thrombin from prothrombin and may act as a regulatory protein[133]. Recent studies suggest that Factor V has a single heavy chain (mol. wt. 125,000) and two light chains (mol. wt. 73,000) which are split during thrombin activation[242]. Factor V and Factor Xa are probably adsorbed to the phospholipid where they act as a complex to convert prothrombin.

Prothrombin or Factor II is a single chain glycoprotein (mol. wt. 70,000), the aminoacid sequence of which has been fully elucidated[304]. Activation of prothrombin by Factor Xa-Factor V complex involves the release of various fragments. Removal of the two "Kringle" structures, Fragments 1 and 2 (Fig. 2.5), releases prothrombin which is cleaved by activated Factor X to produce the two chain molecule thrombin. Thrombin itself is involved in splitting Fragment 1 from the prothrombin molecule (Fig. 2.3) and this may represent a negative feedback control of thrombin

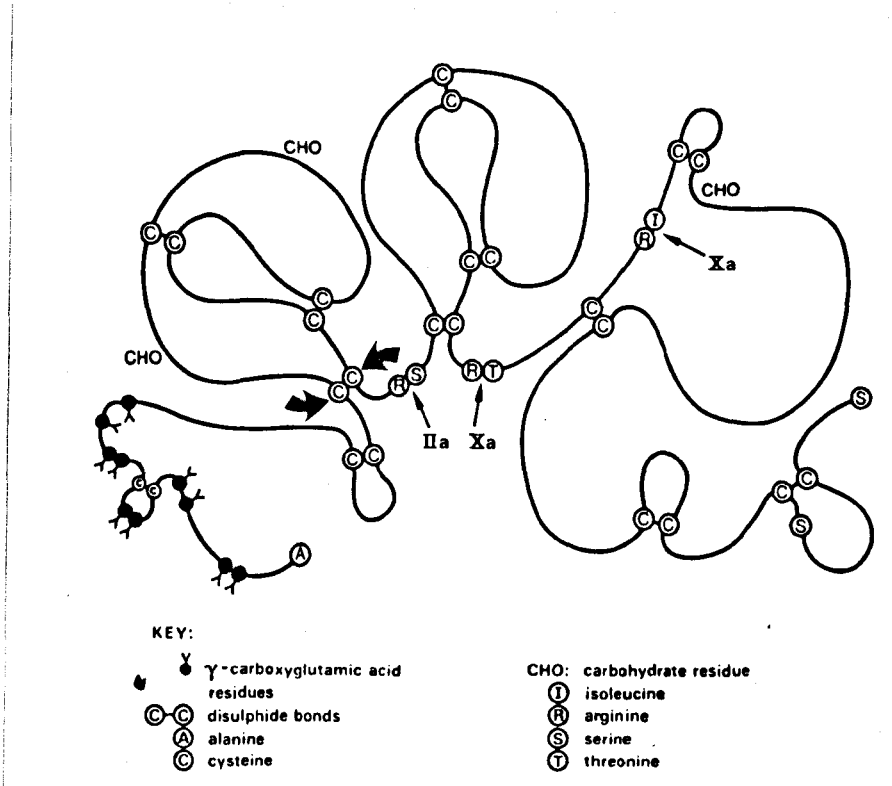


Figure 2.5 The secondary structure of prothrombin (after Magnusson et al[304]). The length of chain representing the Kringle structures (two are shown) is highlighted between the unmarked arrows. The structures are so called due to their resemblance to a Swedish cake. Cleavage points for Factors IIa and Xa are shown.

production. Indeed, in the presence of thrombin inhibitors, the rate of prothrombin conversion can be greatly increased and it has been suggested that Fragment 1 competitively inhibits prothrombin conversion[406] and that Factor V may function by counteracting this inhibitory effect of Fragment 1[133].

Thrombin, therefore, has two chains, a long B chain and a short A chain joined by a disulphide bridge. It is a serine protease with trypsin-like activity, but is distinguished from the other serine proteases such as pancreatic enzymes by the presence of the short A chain. Thrombin activity on fibrinogen has been reported to be highly specific, occurring at four arginyl-glycyl bonds and two arginyl-valine bonds[55]. Recent kinetic studies indicated that this activity occurs within the first 23 amino-acids of the fibrinogen molecule[218]. Thrombin is not only directly involved in the conversion of fibrinogen to fibrin, but also potentiates the activity of Factors VIII and V and converts Factor XIII to its active form (see later). In addition, thrombin can digest fibrin in vitro and produce fibrin degradation products similar to plasmin-released peptides[243] Arginyl-lysine bonds are of considerable importance in this reaction.

The final stage in fibrin production is initiated by the enzymatic action of thrombin upon fibrinogen. Fibrinogen is a soluble plasma protein with a molecular weight of 340,000 composed of two monomeric units, each containing an Aα, Bβ and γ chain with molecular weights of 63,000, 56,000 and 47,000 respectively[171] (Fig. 2.6). These chains are joined by disulphide bridges[323], as are the two monomers, through pairs of α and γ chains. Although usually described as a rod-shaped molecule, electron microscopical evidence suggests that the molecule is spherical or globular, with a diameter of 24 nm[346]. In addition, although fibrinogen is considered as a symmetrical molecule, there is accumulating evidence of heterogeneity within the molecule, particularly of the γ chain[348].

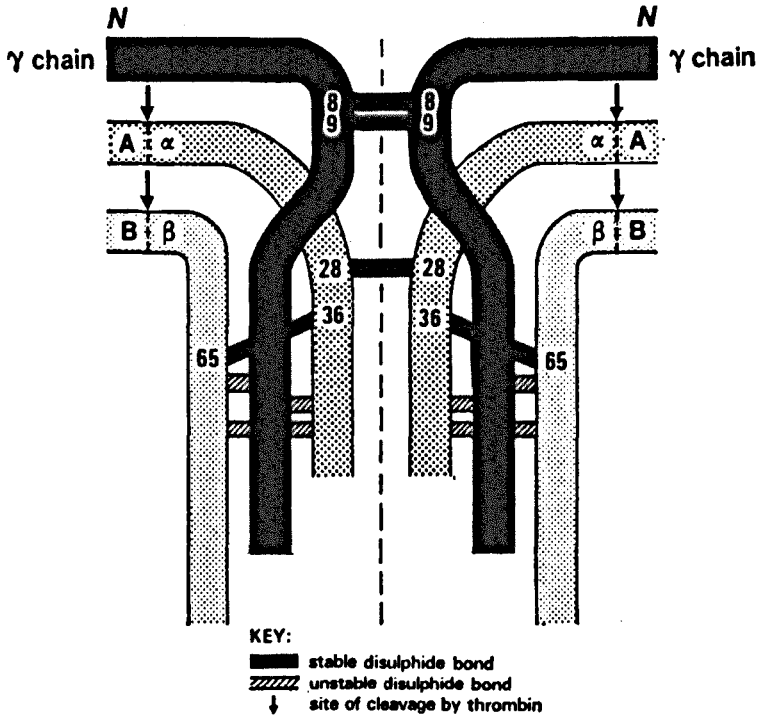


Figure 2.6 Schematic model of the N-terminal disulphide knot (N-DSK) portion of the dimeric fibrinogen molecule (after Blomback and Blomback[54]).

Indeed, Mosher and Blunt[344] have suggested that there may be 36 different forms of bovine fibrinogen.

Thrombin releases fibrinopeptides A and B from the Aα and Bβ chains, thereby producing fibrin monomer (FM). Release of fibrinopeptide A may be associated with a conformational change in the molecule, which not only facilitates the end to end alignment of the fibrin monomers, but makes the B peptide more accessible to thrombin activity. Side-to-side association of the molecules occurs following the release of fibrinopeptide B[54].

The conversion of chains of FM to fully cross-linked fibrin is achieved through the activity of Factor XIII which itself is activated by thrombin. Factor XIII is a transamidase enzyme which occurs in plasma and platelets. Plasma Factor XIII is composed of two A and two B subunits whereas platelet Factor XIII, which is of separate origin, only has two A subunits[319,462]. Recent evidence suggests that plasma Factor XIII may receive its subunit chain from platelet Factor XIII[436]. Thrombin acts on subunit A by limited hydrolysis to release a fragment, which is the active principle, fibrinoligase[297]. In addition, thrombin-catalysed removal of fibrinopeptide A is responsible for the unmasking of the fibrinoligase reactive site on the FM molecule[296]. Polymerisation of the FM by this means greatly enhances the elasticity of the clot and reduces its susceptibility to digestion by plasminogen-urokinase[298], although this has recently been disputed (see Chapter 5). In addition, fibrinogen-thrombin interactions are associated with an induction period during which no fibrin polymerisation is detected. It has been suggested on the basis of evidence obtained from laser fluctuation spectrometer studies that this is the result of a reversible complex between fibrinogen and FM which acts as a buffer system, preventing the concentration of free FM from reaching the level necessary to initiate polymerisation, before a

substantial fraction of the fibrinogen has been converted to fibrin[64]. Thus fibrinogen may act as a natural anticoagulant by delaying clotting through complex formation with FM.

Cross-linked fibrin produced by Factor XIII results from the formation of ε-(γglutamyl)-lysine isopeptide bonds between pairs of γ chains to form γ-γ dimers, and α chains to give an α polymer[322]. Thus, crosslinked fibrin can be identified on sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis by the disappearance of the α and γ chains of fibrinogen and the appearance of the γ dimer.

ROLE OF CELLULAR ELEMENTS IN BLOOD COAGULATION

Much of the evidence for the coagulation mechanisms outlined above has been derived from in vitro studies of cell-free plasma or serum. However, it is axiomatic that intravascular and extravascular blood clotting occur in the presence of large numbers of blood cells and that cellular behaviour considerably influences clot formation. Intravascular thrombus formation has long been known to be associated with platelet activity[439], and in recent years there has been an upsurge in interest concerning the thromboplastic activity of leukocytes and monocytes[364]. It is therefore important to define the role of such cells in coagulation mechanisms.

PLATELETS

Platelets occur as small anuclear cell figures in the circulation. They are disc-shaped ellipsoids, 3 μ in diameter and 1 μ in thickness. They derive as split fragments from the cytoplasm of megakaryocytes, which are large, multinucleated precursor cells in the bone marrow. The platelet has been described as having three structural zones related to

function; the peripheral zone, involved in adhesion, the sol-gel zone, which acts as a cytoskeleton and is involved in contraction, and the organelle zone, which is responsible for secretion (see below)[554]. The peripheral zone comprises the plasma membrane with the extracellular glycocalyx or glycoprotein coat and the lining canalicular system that penetrates the platelet substance. The sol-gel zone lies immediately beneath the plasma membrane and contains microtubules and microfilaments which are intimately involved in the shape changes that occur on aggregation[553]. The organelle zone refers to the more central area of the platelet and contains mitochondria and granules. α granules contain lysosomes, cationic proteins, elastase, permeability enhancing factor, chemotactic factor, mitogenic factor, fibrinogen, β thromboglobulin, and dense granules contain ADP, ATP, serotonin and Ca^{2+} ions.

The primary function of platelets is to participate in haemostasis where they undergo a characteristic series of changes on contact with exposed vascular basement membrane[471], and form a major component of the primary haemostatic plug. This platelet response has been shown to combine several features, namely, change in shape, increased adhesivness, release of platelet substances (secretion) and aggregation. Holmsen[220] has described this response as the basic platelet reaction (BPR) and has outlined, as a working hypothesis, three sequential steps in this process, i.e. induction, transmission and execution. Induction occurs when a platelet activating agent reacts with the cell membrane. Transmission of the response occurs on release of an intracellular transmitter, possibly calcium ions, which stimulates an A.T.P.-requiring process (probably actomyosin contraction). The platelet at this stage may be conditioned to perform shape change (Stage 1), to adhere to other platelets (Stage 2), or to release substances from its dense granules (Stage 3) or from its α granules (Stage 4). The stage of conditioning which the

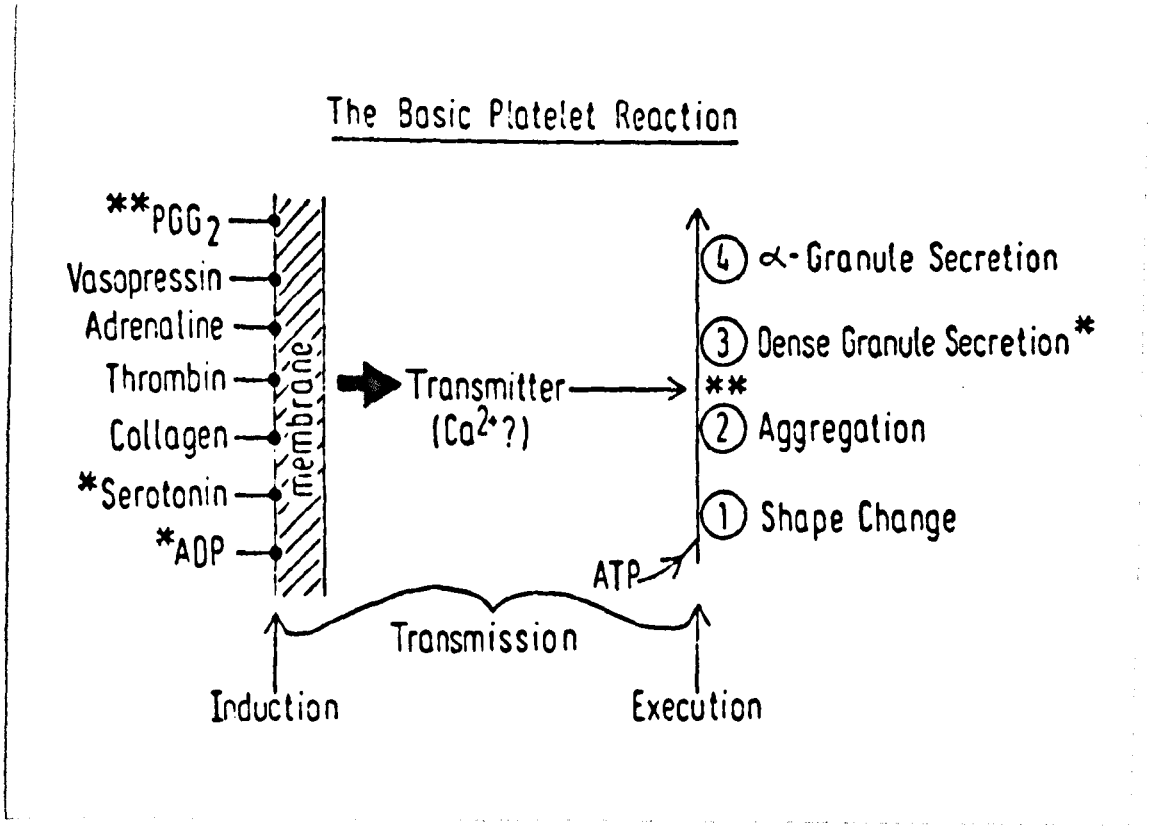


Figure 2.7 Stages of platelet release reaction (after Holmsen[220]).

platelet reaches during transmission is directly determined by the concentration of transmitter and ATP. Execution of the response depends on the occurrence of appropriate internal and external conditions (Fig. 2.7).

Many agents are known to induce platelet activation, including proteolytic enzymes, glycoproteins, particulate matter, bacteria and viruses[352]. However, most studies of platelet activation have centred around a small number of well-known and physiologically important molecules. These include thrombin, collagen, fatty acids, prostaglandins, and their precursors and derivatives, serotonin, adrenaline and noradrenaline and ADP. A central role for ADP in platelet aggregation by all agents was suggested on the basis of in vitro experiments which showed that aggregation did not occur when ADP was removed from the medium[207]. However, it has recently been shown that ADP, although it does cause aggregation[164,165] and is released during the platelet release reaction[226], is not essential for platelet aggregation to occur since collagen, thrombin and the calcium ionophore A23,187 can cause aggregation in the absence of ADP. In addition, the in vitro release of ADP following platelet aggregation may be a laboratory artefact due to hypocalcemia[320]. There is, however, some dispute over this suggestion, most recently put forward by Huzoor-Akbar and Ardlie[231], who deny the effect of hypocalcemia and propose a central role for thrombin in all methods of platelet aggregation.

More recently, attention has been focussed on the prostaglandins and their intermediates, as final mediators of the platelet aggregation response[337]. It is known that, during platelet activation, arachidonic acid is released from the plasma membrane by phospholipase A_2 and rapidly converted into the unstable endoperoxides PGG_2 and PGH_2 (Fig. 2.8)[199] by the enzyme cyclo-oxygenase. These metabolites are potent activators of

platelet activity and in addition may be converted to Thromboxane A_2 (TXA_2) which has a several fold increase in platelet aggregability. TXA_2 is also released by platelet aggregation and although unstable in vitro, is stabilised in vivo by plasma[475]. Conversely, within the vascular endothelium, endoperoxides are converted into prostacyclin PGI_2, which is a potent inhibitor of platelet aggregation[336], and this suggests that prostaglandins have a central role in the control of platelet activation, inhibiting aggregation where it is least desired, i.e. within normal vascular endothelium, and promoting aggregation where it is useful, e.g. within platelets in a haemostatic plug. Both TXA_2 and PGI_2 are thought to influence platelet aggregation by varying the intracellular concentration of cyclic AMP, which is known to have a profound effect on platelet activation[188] (Figure 2.8).

However, it has been shown that thrombin and collagen may activate platelets directly in the absence of both ADP and prostaglandins[70]. In arachidonic acid deprived rats, collagen at high dosage was found to cause aggregation of platelets, even though prostaglandin synthesis was negligible. However, the capacity of platelets to react to threshold doses of collagen depended on the availability of endoperoxides. Thus, although PGG_2 and PGH_2 and TXA_2 are produced in very small quantities in vivo and are extremely short-lived, they serve to amplify the response of the platelet to a given stimulatory agent[454].

Thrombin and collagen are considered to be extremely potent platelet aggregating agents. Although they can act directly on platelets, little is known about the precise mechanism of action. The platelet cell membrane contains thrombin-specific receptors[119], but it is also thought that a hydrolytic enzyme is required to activate platelets[397]. Even less is known concerning the mode of action of collagen. Early speculation concerning an enzyme-acceptor complex between glucosyl-transferase on the platelet

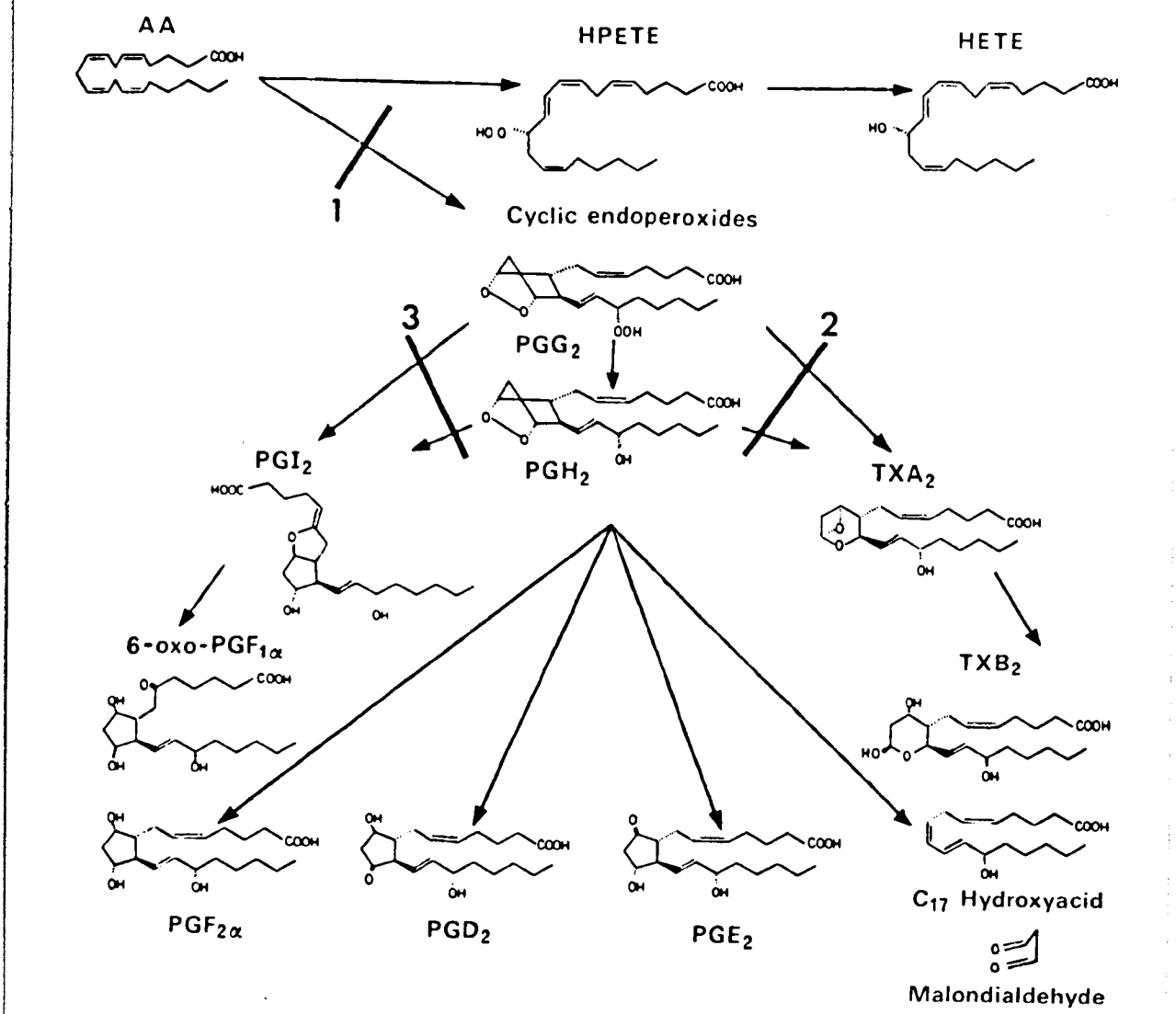


Figure 2.8 Metabolic pathway of arachidonic acid (after Moncada and Vane[337]).

membrane and the galactosyl-hydroxylysine side chains on the collagen molecule[238] is now regarded as untenable since soluble collagen is unable to aggregate platelets. Platelet aggregation occurs only with fibrillar collagen[30,350]. In addition, studies of the effect of genetically distinct collagen types on platelet aggregation (for recent review of collagen biochemistry, see reference 334) have shown that Type III collagen is the most potent platelet aggregating agent[228]. This contrasts with previous work where Type I was considered to be the most active[331]. Other workers have shown that Type I has equivalent activity to Type III[33], but Type IV collagen, of basement membranes, has been reported as the least active[331]. Type III collagen forms thin fibrils and is most abundant in blood vessel walls, whereas Type I occurs as thick collagen fibrils[334]. Finally, the possibility that collagen may cause platelet aggregation via activation of platelet membrane-bound complement has recently been postulated[86]. Complement components C1, C3 and C4 are known to be tightly bound to the platelet membrane[543], and complement consumption by collagen has been demonstrated[497]. In addition, platelets from complement-depleted dogs are unresponsive to autologous collagen[86].

The molecular events which occur in the platelet surface membrane on contact with an aggregating agent are unknown, but certain conditions are required. Ca^{2+} ions are essential and a layer of adsorbed surface protein[127], usually fibrinogen, is necessary[578]. It should also be noted that Factor VIII-related antigen has been proposed as the important adsorbed plasma protein in platelet adhesive reactions, e.g. to vascular endothelium. A requirement for platelet surface glycoprotein (glycocalicin) has also been demonstrated[478]. Not all proteins will promote adhesion; human serum albumin, for instance, is ineffective[331]. An interesting hypothesis, based on physico-chemical mechanisms, has been

put forward to explain the differential adhesive qualities of platelets, as opposed to red cells[313]. In this report, it was shown that adsorbed protein will reduce platelet surface potential and thus promote adhesion. In addition, both ADP at a concentration of 70.5 μg/ml and Ca^{2+} ions also reduce cell surface potential.

In addition to their role in haemostasis, platelets have significant intrinsic procoagulant activity. As stated above, plasma proteins are adsorbed to their surface membrane, and these include several coagulation factors such as prothrombin, fibrinogen and Factors VII, IX, X, V and VIII[212]. Fibrinogen is also contained within the granules and is made available during the release reaction. Although the amount of fibrinogen released is small in relation to plasma fibrinogen, it may provide a high concentration between platelets in an aggregate[552]. Platelet factors 3 (PF 3) and 4 (PF 4) are also released during the BPR. PF 3 is phospholipid and participates in several stages of the intrinsic pathway of coagulation (Fig. 2.2), although it is considerably less active than similar phospholipid obtained from brain tissue[540]. PF 4 has heparin-neutralising activity[113], and may have a role in overcoming any natural heparin-like inhibitors within the system. Platelets can also contribute to the contact phase of clotting[538], by activating Factor XI during collagen-induced platelet aggregation[538], and by catalysing Factor Xa activation[539]. Finally, platelets contain Factor XIII, which is distinct from plasma Factor XIII[319] and assists in the polymerisation of fibrin. It is therefore apparent that wherever platelets are activated in the presence of blood coagulation factors, clotting will take place.

LEUKOCYTES

Studies of the generalised Schwartzman reaction (GSR) have suggested that leukocytes may be associated with clotting activity. The GSR is an

experimentally-induced response to two appropriately spaced sublethal injections of endotoxin, in which widespread intravascular coagulation occurs[186,503]. Since the GSR can be prevented in leucopenic rabbits[144], leukocytes have been implicated as causative agents in GSR-associated clotting mechanisms[364]. In addition, in vitro studies have shown that purified leukocytes obtained from rabbit peritoneal exudates or from whole blood by differential sedimentation, develop significant procoagulant activity (PCA) when stimulated by endotoxin[360]. Unstimulated leukocytes have little or no procoagulant activity[1967]. The activity generated in leukocytes by endotoxin has similar properties to tissue factor activity, since it fails to occur in Factor VII-deficient plasma[291]. In addition, delipidation of leukocyte factor removes its clotting ability, while relipidation restores it[361]. Human leukocytes are less responsive to endotoxin than rabbit cells, and in addition, human leukocytes require the presence of serum[173]. Lymphocytes and macrophages can also generate clotting activity and indeed macrophages appear severalfold more active than polymorphonuclear leukocytes in this respect[173]. The activity appears to be contained within a subcellular microsomal fraction and is tightly bound to the organelle membrane[361].

Other agents that have been reported to induce procoagulant activity in leukocytes include haemagglutinins[432], artificial membranes[364], and simple adhesion to plastic surfaces during culture[257,450]. However, it is uncertain whether endotoxin contamination of these substrates may have induced these responses, since only nanogram quantities are required for leukocyte stimulation[363]. Procoagulant activity in leukocytes can also be stimulated by platelets, or their granules[362] during aggregation and this further emphasises the profound complexity of the inter-relationships between coagulation mechanisms and blood cell behaviour. The recent observation that leukocytes generate thromboplastic activity on adhesion

to vein walls may have some significance in relation to the pathogenesis of deep vein thrombosis[290].

MONOCYTES

Monocytes have been shown to generate procoagulant activity (PCA) after stimulation by endotoxins[438,213]. This activity has been likened to tissue factor activity[518] since it was Factor VII dependent. It has also been shown, however, that simple adhesion of monocytes to foreign surfaces such as glass[518] or latex[467] will lead to cellular PCA. Not all surfaces will activate monocytes in this way; monocytes adherent to a monolayer of cultured endothelial cells produce minimal PCA[518]. Fibrin microclots produced on glass slides by single monocytes have a peculiar stellate appearance which is formed by fibrin crystals radiating out from a central focus occupied by the cell[467]. A clinical analogue of this has been suggested by stellate fibrin formations in the bone marrow of patients with myelofibrosis[359].

A recent report has described the release of PCA from purified cultured monocytes stimulated by guinea pig complement factor C3b[409]. Again, the PCA was similar to tissue thromboplastin in that it required Factor VII and was rapidly inactivated by monospecific antiserum against tissue thromboplastin. Thromboplastin production by these cells was closely related to protein synthesis[408]. This link between coagulation, blood cell behaviour and complement activation further exemplifies the diverse integration between these various biological systems. Future studies of these inter-relationships should prove most fruitful.

NATURAL INHIBITORS OF COAGULATION

No discussion of coagulation mechanisms is complete without

reference to inhibitors of coagulation. Several naturally-occurring inhibitors have been identified, the earliest discovered probably being heparin[325]. Others include the antithrombins, α_2 macroglobulins, α_1 antitrypsin and C_1 esterase, all of which are proteins or their derivatives from plasma.

HEPARIN

Heparins are classified within a group of long chain amino-sugars known as glycosaminoglycans (GAG) which occur ubiquitously throughout mammalian and non-mammalian tissues. Heparins differ from other GAGs in that their intersaccharide linkages are exclusively 1-4, that they contain both D-glucuronic and L-iduronic acids, that the sulphation of these sugars is specific for heparin, and that the glycosamine residue is exclusively D-glucosamine[36]. Anticoagulant properties, at least in vitro, are associated only with heparin among the GAGs and are related to its highly specific structural characteristics[222]. In plasma heparin is completely bound to proteins by direct electrostatic interaction or by other noncovalent mechanisms[281]. Heparin acts by potentiating the action of antithrombin III[112,143](see below). Heparin also reacts directly with thrombin, which may make it more accessible to antithrombin III[36]. Several cell types, notably basophil leukocytes and mast cells, contain heparin within their granules and such cells probably act as "anticlot" cells in contrast to the "clot" cells such as monocytes[467]. There is also considerable heterogeneity among the different tissue heparins[36] and, in spite of technical difficulties with standardisation of heparin assays, there appears to be significant variations in activity[252].

ANTITHROMBINS

Antithrombins, as presently described, are poorly characterised; of six known antithrombins, only three have been shown to function physiologically, i.e. antithrombins I,II and III. Antithrombin I is associated with the reversible removal of thrombin from solution by its adsorption onto fibrin[254], but is probably not of great physiological importance. Antithrombin II is also known as plasma heparin cofactor[411] but it now appears that its activity, if not its structure is identical to that of antithrombin III, since the addition of a specific antibody to antithrombin III removes all the heparin cofactor activity from plasma[136]. Antithrombin III is a α_2 globulin with a molecular weight of 65,000 and its mode of action is to bind irreversibly to thrombin, thereby inactivating both components[573]. Antithrombin III also competitively inhibits activated Factor X, but less rapidly than thrombin-inhibition[1]. It appears that activated Factor Xa is protected from antithrombin III activity during prothrombin conversion by its continued adsorption to phospholipid[234]. Other inhibitory effects of antithrombin III have been reported in relation to Factor IXa, XIa and plasmin[112], but these have not been confirmed. Antithrombin III may also inactivate Factor VII[181].

α_2 MACROGLOBULIN

α_2 macroglobulin is a plasma protein, composed of two monomers, each with a molecular weight of 380,000. It inhibits several proteinases including thrombin, plasmin and kallikrein[35]. Inhibition of thrombin is time-dependent and is not enhanced by heparin. Its mode of action is not precisely known, but it appears that an active fragment

(molecular weight 85,000) is split from the subunit chains of the molecule[202].

α_1 ANTITRYPSIN

α_1 antitrypsin is a glycoprotein with molecular weight about 50,000 and has broad specificity. α_1 antitrypsin has been reported to have about 25% of plasma antithrombin activity, but this has been disputed[286]. Its role in preventing clotting is therefore unclear, although it has been shown to be a powerful inhibitor of Factor XIa[209]. α_1 antitrypsin and α_2 macroglobulin also occur in platelets, although in relatively smaller concentration when compared to platelet-associated fibrinogen or Factor VIII antigen. It is possible that these inhibitors modulate protease-mediated events during the formation of haemostatic plugs and thrombi[354].

C1 INACTIVATOR

C1 inactivator is a glycoprotein of molecular weight 105,000 which inhibits C1 esterase, Factor XIIa and XIa, kallikrein and plasminogen activator[204,318]. Thus it may function in the early phases of intrinsic and extrinsic coagulation.

An as yet incompletely identified inhibitor of Factor XIa with a molecular weight of approximately 65,000 has also been reported, but so far appears to be specific for Factor XIa[367].

SUMMARY

Current concepts of blood coagulation mechanisms have expanded considerably as a result of recent advances in the identification and isolation of newer clotting factors. It is now recognised that the various clotting systems, which are reviewed in this chapter, are closely linked and that activation of one system may trigger coagulant activity in another system. It is therefore likely that blood clotting in the tissues occurs by more than one mechanism, although extrinsic system coagulation via tissue thromboplastin is regarded as the predominant one. In this thesis, an attempt is made to identify the mechanisms of blood clotting in the vitreous of the eye. Each system is not investigated in depth, since this is outside the scope of the thesis, but by studying the clotting activity of plasma in the presence of vitreous and its components, it has been possible to formulate a hypothesis on the most probable mode of intravitreal blood clotting.

CHAPTER 3

MATERIALS AND METHODS USED FOR STUDY OF THE IN VITRO INTERACTION BETWEEN BLOOD AND VITREOUS

INTRODUCTION

This chapter comprises three sections. The first section contains details of the methods used to extract vitreous humour from enucleated eyes. In the second section, techniques for the study of the effects of vitreous on coagulation mechanism are described. The third section deals with the role of platelets in clotting mechanisms within the vitreous. An experimental system was devised to determine whether significant fibrin formation occurred within intact vitreous gels solely as a result of platelet activation. The materials and methods used for these experiments are described here.

PROCEDURE FOR EXTRACTING VITREOUS

It has been observed that the vitreous humour of most species exists as a gel under physiological conditions (Chapter 1). However, a variable proportion of normal adult vitreous consists of a viscous fluid, particularly its central part. The ratio of gel to fluid compartments varies considerably according to the species and the age of the specimen[23]. The vitreous of animals such as cattle and sheep is almost entirely gel, while that of the owl monkey is completely fluid (Chapter 1). Human and rabbit vitreous occupy an intermediate position. Consequently, methods for obtaining samples of vitreous have been adapted to its physical state. Where it is predominantly fluid, samples can be obtained by simple aspiration. When the vitreous is predominantly gel-like, special techniques for its dissection have to be used, and a number of these have been described[20,28,29,198,491].

A method commonly employed to circumvent these problems is to dissect the vitreous in the frozen state[26,42,125,158,195]. Such methods

have been used to study topographical variations in its components[25] and to ensure maximum yield of tissue with minimal contamination from surrounding ocular structures. It should be noted, however, that freezing techniques have been shown to produce artefactual variations in the distribution of soluble macromolecules within the vitreous[491] and should therefore not be used for topographical studies.

In the present investigation, vitreous samples were obtained by three methods:-

(a) an aspiration technique

(b) a dissection technique

(c) a freezing technique

Each method was applied to different studies according to the requirements of the experiment.

MATERIALS

Vitreous samples from a variety of species were used in the preliminary experiments described in this thesis. This was considered necessary in view of the considerable species differences in the concentration of vitreous tissue components[21]. It was found, however, that species differences in the thromboplastic and fibrinolytic properties of the vitreous were not marked. Consequently, bovine vitreous was used for a majority of the in vitro studies, while rabbit eyes were selected as the most suitable for in vivo studies.

Rabbit and dog eyes were obtained from animals which had been used for experiments unrelated to the present studies. In none of these experiments were anticoagulants used. The eyes were normal by gross examination. Cattle and sheep eyes were obtained from the slaughterhouse. All animal eyes were obtained immediately after death and

transported on ice to the laboratory within one hour. Human eyes were obtained from donor cadavers within 12 hours of death. Eyes from subjects who died from haemorrhagic or thrombotic diseases were rejected.

METHODS

Aspiration technique

This method was applied to human and dog eyes. The eyes were cleaned and all extraocular tissues removed. Samples of fluid vitreous were taken by aspiration through a 19 gauge needle inserted through the sclera, according to the method of Walker and Patrick[533]. In some cases, a 25 square millimetre window of sclera, choroid and retina was removed in layers and the vitreous aspirated by a modified "open sky" technique. This procedure was adopted to ensure absence of contamination from other ocular tissues. Using this technique, a maximum of 60% of the total vitreous volume was obtained.

Dissection technique

This procedure was used for cattle and sheep eyes only, since these eyes have few vitreo-retinal adhesions[576]. The eyes were placed, cornea upwards, in eyecups and a 5mm incision was made in the ora serrata with a razor blade. Curved dental forceps and blunt scissors were used to extend the incision to 360° and the anterior segment, lens and vitreous were removed as one piece from the tissues of the posterior segment. With the eye suspended in the vertical position, deep incisions were made with clean scissors into the vitreous gel, tangential to the surface of the lens. Pieces of gel vitreous were allowed to drop into a glass beaker, and were gently homogenised by aspiration through a 20 ml syringe. Vitreous

gels from individual sheep eyes were stored as separate samples, but those from cattle eyes were pooled. Contamination from other ocular structures was minimal. More than 90% of the vitreous was recovered by this technique.

Freezing technique

This procedure was applied mainly to rabbit and human eyes. The eyes were cleaned, and the aqueous humour aspirated by puncturing the eye at the corneo-scleral limbus. Aqueous samples were stored at -20°C. The eyes were then placed in polythene bags and immersed in a solution of CO_2 snow ("dry ice") in acetone (-40 to -60°C) for sixty seconds. The outer coats of the eye were dissected in small pieces with a Thiersch skin-graft blade. As the globe gradually thawed from the periphery to the centre during this procedure, the frozen vitreous could be "shelled out" of the eye in the later stages as a single ice ball. In the same way, the lens could be shelled out of the "patellar fossa" of the frozen vitreous. The entire vitreous was recovered from the eye by this method and was free of contamination by other structures.

EFFECT OF VITREOUS ON BLOOD COAGULATION MECHANISMS

Experiments were designed to test the effect of vitreous on coagulation mechanisms. In general standard clotting tests, or modifications thereof, were used and any changes induced in the system by the presence of vitreous were observed.

MATERIALS

Vitreous samples from human, dog, sheep, rabbit and cattle eyes were obtained by one or more of the above methods in each case.

Whole blood was obtained from healthy human volunteers with 0.13M sodium citrate or 1% EDTA (di-sodium salt) as the anticoagulant. Platelet-poor plasma (PPP) was prepared by centrifugation of the blood at 3000 r.p.m. for 20 minutes. Platelet-rich plasma (PRP) was prepared by centrifugation of the blood at 4000G for five minutes. The plasma samples were used immediately after preparation or stored at -20°C. Non-activated platelet-poor plasma was obtained from whole blood by clean venepuncture. All procedures were performed at 0-4°C. The first two mls of blood was discarded and the remainder was collected directly through a cannula into siliconised plastic tubes. The samples were immediately centrifuged at 11,000 r.p.m. for 30 minutes, and the plasma withdrawn through siliconised Pasteur pipettes. Non-activated plasma was stored at -80°C unless used immediately. Serum was obtained from clotted human blood samples after centrifugation at 3000 r.p.m. for 20 minutes.

Calcium chloride was dissolved in distilled water to a final concentration of 0.04 or 0.025M. Calcium phosphate was used as a 0.5 per cent solution.

Phosphate-buffered saline contained 0.2M Sodium phosphate in 0.15M sodium chloride, pH 7.4.

Tris-buffered saline contained 0.05M tris (hydroxymethyl) aminomethane-HCl and 0.01M NaCl, pH 7.4.

Trisodium citrate was used as a 3.9 per cent solution in distilled water.

Tissue thromboplastin was obtained from Hyland Laboratories, California.

Cephotest was supplied by Nyegaard and Co. A/S, Oslo, Norway.

Coagulation factor-deficient substrate plasmas were obtained from Dade Diagnostics Inc., Miami. Coagulation factor concentrates containing

Factors II, IX and X were prepared according to the method of Middleton et al[353] from Factor VIII-deficient plasma. The samples (Defix) were supplied by the Protein Fractionation Centre, Royal Infirmary, Edinburgh. A similar protein concentrate, Factor VIII-Inhibitor Bypassing Activity (FEIBA), which contained Factors II, IX, X and VII, plus other unidentified activated components, was obtained from Immuno AG, Vienna, Austria.

Ecarin was obtained from Sigma Ltd., London.

Fibrinogen (human) was obtained from Kabi Pharmaceuticals Ltd., Stockholm, Sweden. It contained 90% clottable protein. Kabi also supplied the chromogenic peptide substrates S2302 (H-D-Pro-Phe-Arg-pNa) and S2222 (Bze-Ile-Glu-Gly-Arg-pNa). S2302 was prepared as a 2mM stock solution in buffer (Tris-HCl 50 mM, NaCl 12mM, pH 7.8) while S2222 was prepared at the same concentration in buffer (Tris-HCl 50mM, NaCl 0.15M, pH 7.4). Aliquots (0.5 ml) were stored at -80°C.

Platelet substitute (Platelin) and Russell's viper venom-cephalin were obtained from Diagen Labs., U.K.

Thrombin (bovine) was obtained from Parke Davis, Detroit, Michigan.

Arvin was supplied by Berk Pharmaceuticals Ltd., Shalford, Surrey.

Hyaluronic acid was obtained as the sodium salt, from Miles Laboratory, London. It was prepared by salt precipitation from human umbilical cords and contained $<0.3\%$ protein. Its intrinsic viscosity (η) varied between 59 and 62, corresponding to a molecular weight of $(4\text{-}5 \times 10^5)$[92].

Dialysis casings were obtained from Visking and Co., Chicago, Illinois, and prepared by boiling in glass distilled water.

Collagen suspensions were prepared from equine tendon collagen

and supplied by Hormon-Chemie, Munich.

Bentonite was a gift from Dr. J.-M. Lavergne, Paris.

Diethyl-amino ethane A50 (DEAE)-sephadex was obtained from Pharmacia, Sweden.

METHODS

The effects of vitreous and its components on blood coagulation mechanisms were studied in glass or plastic tubes of 8 mm internal diameter, unless otherwise stated. Centrifugations were performed in an MSE-6L centrifuge at 0°C to 4°C. Ultracentrifugations were performed in a Beckman L5-50 ultracentrifuge at 0° to 4°C.

Preparation of vitreous samples

Preliminary studies of vitreous thromboplastic activity were performed using untreated samples of fluid vitreous. Further studies were performed using collagen-free vitreous prepared by ultracentrifugation of the vitreous at 65,000G for 1 hour at 4°C. The supernatant, which contained hyaluronic acid and soluble macromolecules, was dialysed for 24 hours against 100 volumes of Tris-buffered saline, pH 7.4, and stored at -80°C. The pellet, which comprised mainly vitreous collagen, was prepared as a collagen suspension in Tris-buffered saline, pH 7.4, by sonication in an MSE ultrasonic disintegrator (PC-495) at medium power, amplitude 3, for 15-45 minutes. Bovine and rabbit vitreous samples only were used for collagen suspensions.

Recalcification time of plasma in presence of vitreous

Thromboplastic activity of the vitreous was studied by recording

the recalcification time of fresh platelet-poor plasma in the presence of vitreous or its separated components. Various modifications of the basic system were studied using substrate deficient plasma and Tris-saline controls (see Chapter 4). Recalcification times were performed in replicates of at least six in a 37°C water bath. The effects of vitreous and its components on the recalcification of non-activated plasma were also studied.

Assay of Factor XII activity in vitreous via prekallikrein activation

This assay was modified from the method described by Mattler and Bang[316] for the detection of prekallikrein in samples of plasma using chromogenic substrate S2302. The assay is based on the conversion of prekallikrein to kallikrein in the presence of activated Factor XII and high molecular weight kininogen. Kallikrein then releases p-nitroaniline (pNa) from the chromogenic substrate S2302 as follows:-

1. Factor XII $\xrightarrow[\text{Contact}]{}$ Factor XIIa

2. Prekallikrein $\xrightarrow[\text{High molecular weight kininogen}]{\text{Factor XIIa}}$ kallikrein

3. H-D-Pro-Phe-Arg-pNa + H_2O $\xrightarrow{\text{kallikrein}}$ H-D-Pro-Phe-Arg-OH + pNa

The test system used in the present study comprised the following:-

0.7 ml buffer (50 mM Tris HCl: 12mM NaCl, pH 7.8)

0.1 ml Factor XII deficient plasma

0.1 ml sample (vitreous)

0.1 ml chromogenic substrate (S2302) from stock solution

0.1 ml cephotest

In this system assay, prekallikrein and high molecular weight kininogen were provided by the Factor XII-deficient plasma, and therefore any release of p-nitro-aniline (pNa) would occur as a result of contact activation of Factor XII which was present in the sample. Contact activation in the test system was achieved by cephotest. Negative controls were included by omitting the cephotest. The release of pNa was measured spectrophotometrically at optical density A^{405} in plastic cuvettes at $37^{o}C$. The time course of pNa release was plotted. Factor XII activity in vitreous was compared with that of normal plasma.

Assay of Factor Xa activity in vitreous

Factor Xa activity can be measured in coagulation factor concentrates by a similar method using chromogenic substrate S2222. The method has been described by Pepper et al (1977) and is used for screening Factor IX concentrates for thrombogenic activity. The principle is based on the release of pNa from S2222 by activated Factor X:-

1. Bze-ILE-Glu-Gly-Arg-pNa + H_2O

Factor Xa →

Bze-ILE-Glu-Gly-Arg-OH + pNa

In the present system, the assay was modified to detect any Factor Xa or Factor Xa-enhancing activity in vitreous samples. The test system was as follows:-

0.1 ml Defix (coagulation factor concentrate containing Factors II, IX and X)
0.1 ml sample (vitreous)
0.1 ml platelet substitute
0.5 ml buffer (0.5M Tris HCl, 0.15M NaCl)
0.2 ml chromogenic substrate (S2222)

The samples were incubated for five minutes at 37°C and the A^{405} was read at two minute intervals to detect any intrinsic Factor Xa activity. A volume (0.05 ml) of 0.04M $CaCl_2$ was then added to the samples and A^{405} read as before. Any alteration in the generation of Factor Xa in the samples was detected by a shift in the time-course curve of pNa release.

Assay of Factor VII-enhancing activity in the vitreous

An identical system was used to detect Factor VII activity in the vitreous. However, in this case the coagulation factor concentrate (FEIBA) contained Factors II, IX, X and VII, plus other unidentified activated components. Thus, vitreous was tested for its ability to enhance or diminish Factor VII activity within the system.

Assay of prothrombin-converting activity in the vitreous

Prothrombin-converting activity in the vitreous was assayed using the following test system; 0.1 ml purified prothrombin (20% normal plasma activity), 0.1 ml Factor V (25% normal plasma activity), 0.1 ml human fibrinogen (2.4 mg/ml) and 0.1 ml platelet substitute were mixed with 0.1 ml dialysed bovine vitreous or Tris-buffered saline, pH 7.4. A volume (0.1 ml) of 0.04M $CaCl_2$ was added to the mixture and the clotting time recorded. Ecarin at various concentrations was used as a positive control in a system which contained 0.1 ml ecarin, 0.1 ml prothrombin and 0.1 ml fibrinogen. It has previously been shown that ecarin has direct prothrombinase activity[264].

Preparation of prothrombin

Prothrombin was prepared from human blood by the method currently used by Dr. J.-M. Lavergne (personal communication), which is based on the method described by Weilland and Soulier[547]. 300 mls of plasma were obtained in 1% EDTA and added to 54 mls of 0.6 bentonite slurry (35 g/L, prepared 24 hours previously and kept at 4°C). The mixture was agitated for 15 minutes at 0°C, and centrifuged at 3000 r.p.m. for 25 minutes at 4°C. The procedure was repeated several times using decreasing concentrations of bentonite until all Factor X activity (less than 0.01%) was removed. 300 mls of bentonite adsorbed plasma was then further adsorbed with 0.5% calcium phosphate, and the mixture agitated at 3000 r.p.m. for 25 minutes at 4°C. The supernatant was discarded and the calcium phosphate washed twice in normal saline, using a volume of fluid equivalent to the volume of the discarded plasma. Prothrombin was then eluted from the calcium phosphate by agitation in a 1 in 20 volume of trisodium citrate for 15 minutes. After centrifugation at 3000 r.p.m. for 25 minutes, the supernatant was removed and the elution procedure repeated with a 1 in 40 volume of trisodium citrate. The supernatants from both elutions were recombined and assayed for Factor II and X (see below). The citrate elutions were dialysed against 0.2M sodium phosphate buffer in 0.15M sodium chloride, pH 7.4, at 4°C. The samples were applied to a 2.6 x 17 cm DEAE-sephadex A50 column and eluted in a NaCl gradiant, 0.15M-0.7M in starting buffer. Fractions containing prothrombin activity were pooled and tested for homogeneity by electrophoresis on 7.5% polyacrylamide gels containing sodium dodecyl sulphate (see Chapter 6). Homogeneity was confirmed by the presence of a single band.

The activity of the prothrombin was assayed by comparing the effect

of prothrombin, diluted 1 in 20, on the recalcification time of Factor II deficient plasma in the presence of tissue thromboplastin, with the effect of normal plasma at the same dilution. Factor X activity was similarly assayed using Factor X deficient plasma, in the presence of Russell's viper venom and platelet substitute.

Preparation of Factor V

Crude Factor V was prepared from human blood by the method of Breckenridge and Ratnoff[65]. Human plasma was obtained in 1% EDTA, adsorbed three times with 10 mg calcium phosphate per ml of plasma and centrifuged at 2100G for 10 minutes after each adsorption. The supernatant plasma was then fractionated at 4°C with a neutral solution of saturated ammonium sulphate by dropwise addition of the ammonium sulphate to the plasma until the desired concentration was reached. After incubation for fifteen minutes at 40°C, the mixture was centrifuged at 2000G for 10 minutes. The protein fraction which was soluble at 25% saturation but insoluble at 50% saturation was dissolved in a volume of distilled water equal to 1/20 of the original plasma volume. It was then dialysed against running tap water for 20 minutes until clear, and then against a 0.1M sodium acetate buffer containing 0.01M calcium chloride, pH 5.4, for 24 hours at 4°C. The precipitate then formed was recovered by centrifugation and stored at -80°C. Factor V activity in the sample was assayed by the recalcification time of Factor V deficient substrate plasma in the presence of tissue thromboplastin. Prothrombin activity was also tested on Factor II deficient plasma as above, and was found to be minimal.

CLOTTING STUDIES IN THE PRESENCE OF VITREOUS GEL: THE ROLE OF PLATELETS

The experiments described in this section were designed to evaluate the role of collagen, which is the gel-forming component of the vitreous[21], in generating procoagulant activity via the activation of platelets.

MATERIALS

These are described above. Other materials included sodium dodecyl sulphate, β mercaptoethanol and crystalline urea, all supplied by British Drug Houses, Poole, U.K.

METHODS

Platelet aggregation

The aggregation of human platelets by vitreous collagen was studied by the method of Born and Cross[59], using a Bryston Platelet aggregometer (Brown Ltd., Leicester, U.K.). 0.1 ml platelet-rich plasma was incubated with samples of vitreous collagen suspension, equine tendon collagen or saline at 37°C, and the aggregation of the platelets recorded on a Bryans 27,000 chart recorder.

Fibrin formation in platelet aggregates within vitreous gels

The following experiment was devised to determine whether fibrin formation could occur in vitreous gel solely as a result of platelet activation.

Fresh bovine vitreous was dissected from cattle eyes and pieces of

intact gel were placed in 20 ml glass universal containers or 15 ml transparent ultracentrifuge tubes. Fresh citrated platelet-rich plasma (0.5 ml) was slowly injected into the centre of the gel and clot formation assessed visually. The samples were allowed to stand for 30 minutes at room temperature. The presence of fibrin in the samples was sought using histochemical techniques and polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS).

Histochemical technique

Samples of vitreous gel with platelet-rich plasma were fixed for 24-48 hours in formalin. To avoid disrupting the gel, the fixative was gently layered onto the surface of the vitreous in the universal glass containers. The samples were passed through graded alcohols, and conventional methods of wax embedding and staining with Martius Scarlet Blue (MSB) for fibrin were used (see Chapter 11).

S.D.S. polyacrylamide gel electrophoresis

Samples of vitreous gel with platelet-rich plasma in ultracentrifuge tubes were spun at 65,000G for one hour at 0-4°C. The pellets were washed for 24 hours with 0.85% saline and dissolved in 1 ml of 8M urea. To each sample, 0.1 ml of a ten per cent solution of sodium dodecyl sulphate plus the same volume of ten per cent β-mercaptoethanol were added. The samples were incubated for 24-48 hours at 37°C and run on 7.5% polyacrylamide gels. The preparation of polyacrylamide gels used for fibrin analysis is described in Chapter 6.

CHAPTER 4

STUDIES ON THE MECHANISM OF BLOOD CLOTTING IN THE VITREOUS

INTRODUCTION

In the review of blood coagulation outlined in Chapter 2, several mechanisms are described whereby clotting of whole blood may be initiated. These include the intrinsic coagulation mechanism which utilises humoral plasma factors, the extrinsic coagulation mechanism in which tissue thromboplastin is required, and cellular mechanisms which involve activation of platelet or leukocyte procoagulant activity.

Clotting in the tissues occurs by means of the extrinsic system, and is dependent on the release of thromboplastic activity from injured tissues. The level of thromboplastic activity varies with the tissue, and is particularly high in lung, brain and placenta[556, 557]. The vitreous represents an unusual connective tissue in many respects (see Chapter 1), but clinical and experimental studies indicate that rapid clotting occurs after bleeding into the vitreous gel (for review, see reference 43). The precise mechanism of intravitreal blood clotting is less clear. Although early studies suggested that the vitreous had significant procoagulant activity[473], it was later shown that tissue thromboplastic activity of the vitreous was minimal[382, 421]. Regnault and Larrieu[421] speculated on the unique nature of vitreous coagulant activity which, in their studies, was similar to activated Factor X, thereby resembling the venom of the Tiger snake (Notechis scutatus scutatus).

A common feature of these studies was the use of crude vitreous preparations where little attempt was made to control such factors as pH and ionic composition. In addition, contamination from other ocular tissues such as the retina, which is high in thromboplastic activity[582], could not be excluded[473].

More recent experiments on coagulation mechanisms in the vitreous

have suggested that clotting of whole blood is initiated by platelet-collagen aggregation which indirectly triggers the intrinsic coagulation mechanism[106,490]. However, although vitreous collagen was shown to have considerable platelet aggregating activity, no direct evidence for fibrin formation in the vitreous by this means was documented.

The present studies were therefore undertaken to determine which, if any, of these mechanisms was relevant to the process of blood coagulation in the vitreous gel.

MATERIALS AND METHODS

These have been outlined in detail in Chapter 3.

PLASMA SAMPLES

Samples of human platelet-rich plasma, platelet-poor plasma and non-activated platelet-poor plasma were prepared as described in Chapter 3.

VITREOUS SAMPLES

Human, bovine, dog and rabbit samples were used. The human and dog samples were obtained by the aspiration technique, the bovine samples by the dissection technique, and the rabbit samples by the freezing technique. Human and dog samples were used undialysed, but corrected for pH (7.2-7.4). Bovine samples were used for the majority of the coagulation studies. After the removal of the collagen by ultracentrifugation (see Chapter 3), the supernatant vitreous was dialysed against 100 volumes of Tris-saline buffer for 24 hours at 4°C and stored at -80°C. The pellets recovered after ultracentrifugation of rabbit and bovine vitreous

were prepared as collagen suspensions in Tris-buffered saline by sonication. The collagen concentration of rabbit vitreous is approximately twice that of bovine vitreous[21], while the total yield of vitreous gel from the bovine eye was four-fold that from the rabbit. Appropriate volume adjustments for the final suspension in buffer were therefore made to give comparable results.

COAGULATION STUDIES

Recalcification Times of Plasma in presence of Vitreous

These were performed in glass or plastic tubes as described. The recalcification times of normal human plasma and Factor XII, X, VII and II deficient plasmas, in the presence of vitreous or Tris-buffered saline were compared. The standard assay contained 0.1 ml plasma, 0.1 ml vitreous or Tris-saline and 0.1 ml 0.04M $CaCl_2$. The clock was started on the addition of the calcium, and the tubes were gently agitated in a waterbath at 37°C until clotting occurred. Each test was performed in a minimum of six replicate tubes. The effects of collagen-free dialysed bovine vitreous, bovine vitreous collagen suspension, and hyaluronic acid on the recalcification time of non-activated human plasma were also tested. The system contained 0.1 ml plasma, 0.1 ml platelet substitute, 0.1 ml buffer or test solution, and 0.1 ml 0.04M $CaCl_2$.

COAGULATION FACTOR ASSAYS

Assays of Factor XII activity, Factor Xa activity, Factor VII-enhancing activity and prothrombin-convertin activity were performed on samples of dialysed bovine vitreous as described in Chapter 3.

CLOTTING STUDIES IN THE PRESENCE OF VITREOUS GEL: ROLE OF PLATELETS

Platelet aggregation

Platelet aggregation studies were performed in a Bryston platelet aggregometer as described in Chapter 3 using human platelet-rich plasma and rabbit or bovine vitreous collagen suspensions. Equine tail-tendon collagen suspension was used as a positive control.

Fibrin formation in platelet aggregates within vitreous gels

Experiments were devised to detect fibrin by histochemical means and by SDS polyacrylamide gel electrophoresis within platelet aggregates which were induced in vitreous gels, by the injection of 0.5 ml citrated platelet-rich plasma. The experimental model is fully described in Chapter 3. The experiments were performed in triplicate on two separate occasions. Histochemical staining of fibrin employed M.S.B. Normal vitreous and untreated platelet-rich plasma clots were also studied histologically to ensure suitable control data. The detection of the constituent chains of cross-linked and non-cross-linked fibrin by S.D.S. polyacrylamide gel electrophoresis is described in Chapter 6. Samples (0.5 ml) of citrated platelet-rich plasma in vitreous were solubilised with urea, S.D.S. and β-mercaptoethanol after ultracentrifugation as described in Chapter 3, and analysed by polyacrylamide gel electrophoresis for the presence of fibrin. Standard fibrin solutions were also run on polyacrylamide gels as controls. These included thrombin-clotted fibrinogen (0.5 ml of 1% human fibrinogen clotted with 10 u bovine thrombin), thrombin-clotted human platelet-poor plasma (0.5 ml plasma plus 10 u thrombin), and Arvin-clotted platelet-poor plasma (0.5 ml plasma plus 0.1ml 70u/ml Arvin). A sample (0.5 ml) of non-clotted solubilised platelet-rich plasma was also analysed after ultra-

centrifugation in order to provide control data on intrinsic platelet proteins. After solubilisation in S.D.S. and β-mercapoethanol, the samples were diluted one part in fifty with 8M urea before application to the polyacrylamide gels.

Effect of platelet aggregation by vitreous collagen on plasma clotting times

Since the release of procoagulant activity from platelets requires the presence of free calcium ions (see Chapter 2), experiments were designed to compare the clotting time of plasma in the presence or absence of vitreous collagen-aggregated platelets. The experimental system is described in the Results.

RESULTS

COAGULATION STUDIES

No reactions occurred when undiluted vitreous from all species tested was mixed with purified human fibrinogen (1 mg/ml) or bovine thrombin (10 u/ml) in equal volume. In addition, no spontaneous clotting was observed during a period of one hour after fluid vitreous was added to fresh citrated human platelet-poor plasma.

Recalcification Tests

The recalcification time of fresh plasma was unaffected by the presence of human vitreous in glass tubes (Table 4.1a), but there was a slight shortening of the recalcification time of human plasma in plastic tubes when human vitreous was present (Table 4.1b). However, fresh dog vitreous (Table 4.2) and dialysed bovine vitreous (Table 4.3)

Effect of Human Vitreous on the Recalcification Time of Fresh Human Platelet-poor Plasma in Glass Tubes

Sample No.	Recalcification Time (secs) Plasma + Vitreous	Plasma + Buffer
1	136	117
2	150	131
3	90	164
4	91	-
5	100	130
6	97	130
7	140	140
8	170	160
9	94	130
10	91	132
11	115	151
12	190	-
Mean	122	137.7
S.D.	34.5	14.5
S.E.M.	9.9	4.3
P value*	0.18	

TABLE 4.1a

*Values for P were derived using the unpaired Students' t-test in all tables in the thesis, unless otherwise stated.

Effect of Human Vitreous on the Recalcification Time of Fresh Human Platelet-poor plasma in Siliconised Plastic Tubes

	Recalcification Time (secs)	
Sample No.	Plasma + Vitreous	Plasma + Buffer
1	205	240
2	228	236
3	233	375
4	225	330
5	260	264
6	217	316
7	140	251
8	115	352
9	235	314
10	231	292
11	180	-
12	120	-
Mean	199.1	297.0
S.D.	48.8	48.4
S.E.M.	14.1	15.3
P value	.0001	

TABLE 4.1b

Effect of Dog Vitreous on the Recalcification Time of Fresh Human Platelet-poor Plasma in Siliconised Plastic Tubes

Sample No.	Recalcification Time (secs) Plasma + Vitreous	Plasma + Buffer
1	260	278
2	285	299
3	398	301
4	358	317
5	335	277
6	229	265
7	271	-
8	280	-
Mean	302	289
S.D.	56.4	19.3
S.E.M.	19.9	7.9
P value	0.62	

TABLE 4.2

had no effect on the recalcification time of normal human plasma in plastic tubes. In addition, dialysed bovine vitreous had no procoagulant effect on Factor X or Factor II deficient plasma, and even had a slight inhibitory effect on Factor II deficient plasma when compared with a buffer control (Table 4.3). This indicated that vitreous contained little, if any, Factor X or Factor II activity. The recalcification time of Factor VII deficient plasma was significantly prolonged in the presence of vitreous, but the reverse effect was observed with Factor XII deficient plasma, where vitreous caused a marked shortening of the recalcification time (Table 4.3). Purified human sodium hyaluronate at a final concentration of 0.66 mg/ml had no accelerating effect on the recalcification time, and if anything, slightly prolonged the clotting time of both normal and Factor XII deficient human plasma (Table 4.3). However, a significant shortening of the recalcification time of non-activated plasma occurred in the presence of sodium hyaluronate and vitreous (Table 4.4). A time-dependent relationship in this effect could not be demonstrated unequivocally since there was a parallel reduction in the control values (Fig. 4.1). By contrast, suspensions of vitreous collagen failed to cause a shortening of the recalcification time of non-activated plasma (Table 4.4).

COAGULATION FACTOR ASSAYS

Assay of Factor XII activity

The assay of Factor XII activity in samples of bovine vitreous was measured by the release of p-nitroaniline dye (pNa) from the chromogenic substrate S2302 in a system which depended on the activation of prekallikrein by Hageman factor (Factor XII) (see Chapter 3). Figure 4.2

TABLE 4.3

Effect of Bovine Vitreous and Hyaluronate Sodium on the Recalcification Times of Standard Human Plasma

Sample	Normal plasma			Factor XII deficient plasma			Factor VII deficient plasma		Factor X deficient plasma		Factor II deficient plasma	
	Buffer	Vitreous	H.A.*	Buffer	Vitreous	H.A.*	Buffer	Vitreous	Buffer	Vitreous	Buffer	Vitreous
1	133	151	145	716	328	904	109	224	>3,600	>3,600	904	>3,600
2	135	146	142	853	325	857	98	217	>3,600	>3,600	932	>3,600
3	131	120	155	901	324	810	111	256	>3,600	>3,600	921	>3,600
4	128	130	153	887	327	925	103	249	>3,600	>3,600	916	>3,600
5	127	118	159	852	327	970	114	249	>3,600	>3,600	921	>3,600
6	123	132	147	889	323	-	112	261	>3,600	>3,600	949	>3,600
Mean	129.5	132.8	150.2	849.7	325.0	893.0	107.8	242.7	>3,600	>3,600	923.0	>3,600
S.D.	4.4	13.4	6.5	68.4	1.9	61.7	6.1	17.9	-	-	15.3	-
S.E.M.	1.8	5.5	2.66	27.9	0.8	27.6	2.5	7.3	-	-	6.2	-
P value		0.58	<.0005		<.0001	.30		<.0001				

*HA - Hyaluronate sodium (η) 64, concentration 0.66 mg/ml.

TABLE 4.4

Effect of Dialysed Bovine Vitreous, Bovine Vitreous Collagen, and Hyaluronate Sodium on Recalcification Time of Non-Activated Human Plasma

	Recalcification Time (secs)					
Sample	Buffer	Dialysed Vitreous	Buffer	Vitreous Collagen Suspension	Buffer	Hyaluronate Sodium 0.5 mg/ml
1	292	218	208	230	292	241
2	352	214	203	242	352	255
3	403	211	206	240	403	257
4	353	238	208	230	353	270
5	332	232	205	235	332	233
6	314	230	201	231	314	203
Mean	341	223	205.7	234.7	341.0	243.2
S.D.	38.3	10.96	2.8	5.3	38.3	23.6
S.E.M.	15.6	4.48	1.1	2.2	15.6	9.6
P value	$<.0001$		$<.0001$		$<.0005$	

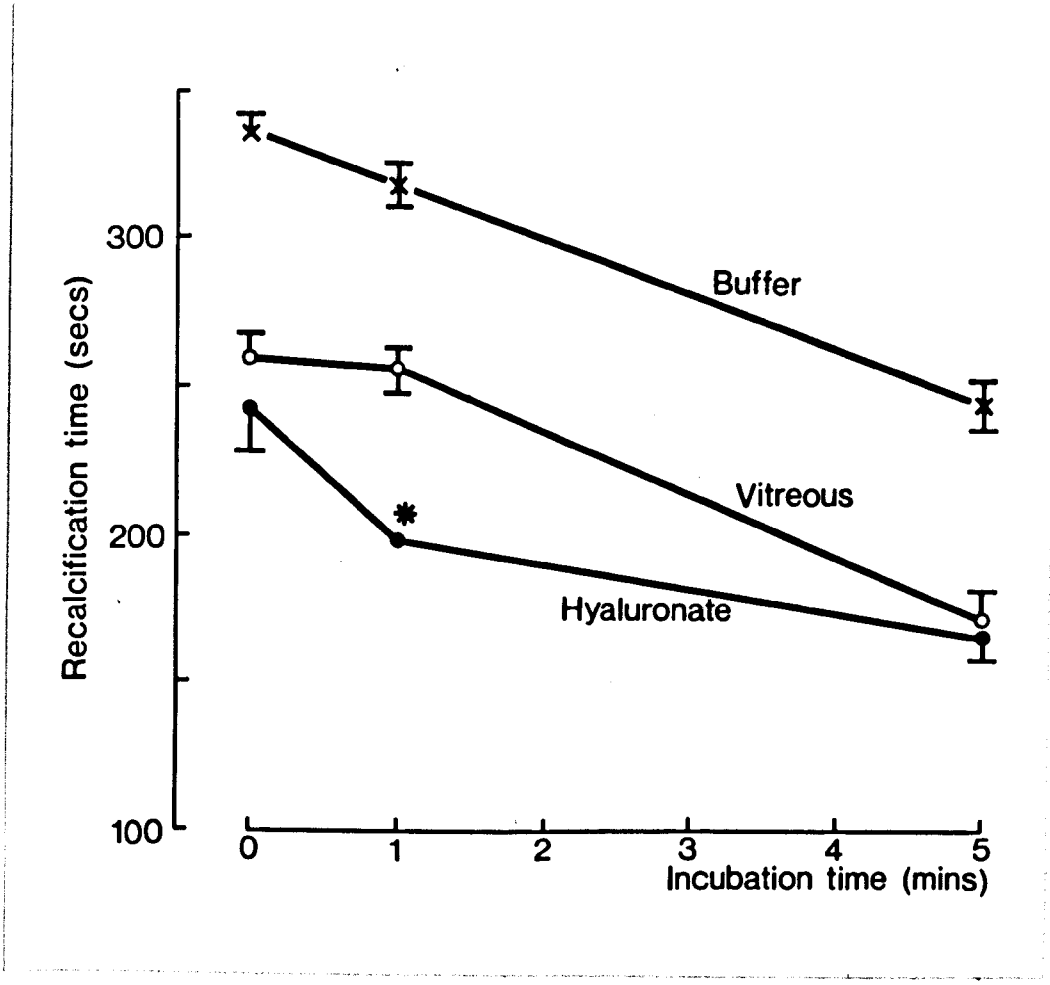


Figure 4.1 Effect of incubating non-activated human plasma with hyaluronate sodium and dialysed vitreous for varying time periods, on the recalcification time of the plasma. Bars represent means plus S.E.M. of six replicate samples; * mean of duplicate samples.

shows that vitreous caused a slow release of pNa as measured by its absorbance (A^{405}) in the presence of the surface activator cephotest. The Hageman factor-like activity of the vitreous was considerably less than that found in plasma diluted one part in ten, as shown by the slow release of pNa by vitreous in the early stages of the reaction (Fig. 4.2).

Assay of Factor Xa activity

Assay of Factor Xa activity in bovine vitreous was determined by the release of pNa from chromogenic substrate S2222 in the presence of a protein concentrate containing Factors II, IX and X (see Chapter 3). No intrinsic Factor Xa activity was detected in vitreous, since pNa release did not commence until Ca^{2+} was added to the samples. In addition, vitreous caused a slight decrease in the rate of pNa release after recalcification as compared to buffer (Fig. 4.3). This indicated that vitreous had a slight inhibitory effect on Factor Xa generation.

Assay of Factor VII-enhancing activity

The assay of Factor VII-enhancing activity in vitreous was tested in identical fashion using a protein concentrate which contained Factors II, IX, X and VII, plus other unidentified activated components (see Chapter 3). No intrinsic Factor VII-enhancing activity was detected in vitreous, nor did vitreous affect the generation of Factor VIIá after recalcification (Fig. 4.4).

Assay of prothrombin-converting activity in the vitreous

A purified system containing prothrombin, fibrinogen, Factor V and platelet substitute was used to identify prothrombin-converting activity in vitreous (see Chapter 3). Three duplicate sets of experiments were run, using dialysed bovine vitreous. Positive (ecarin)

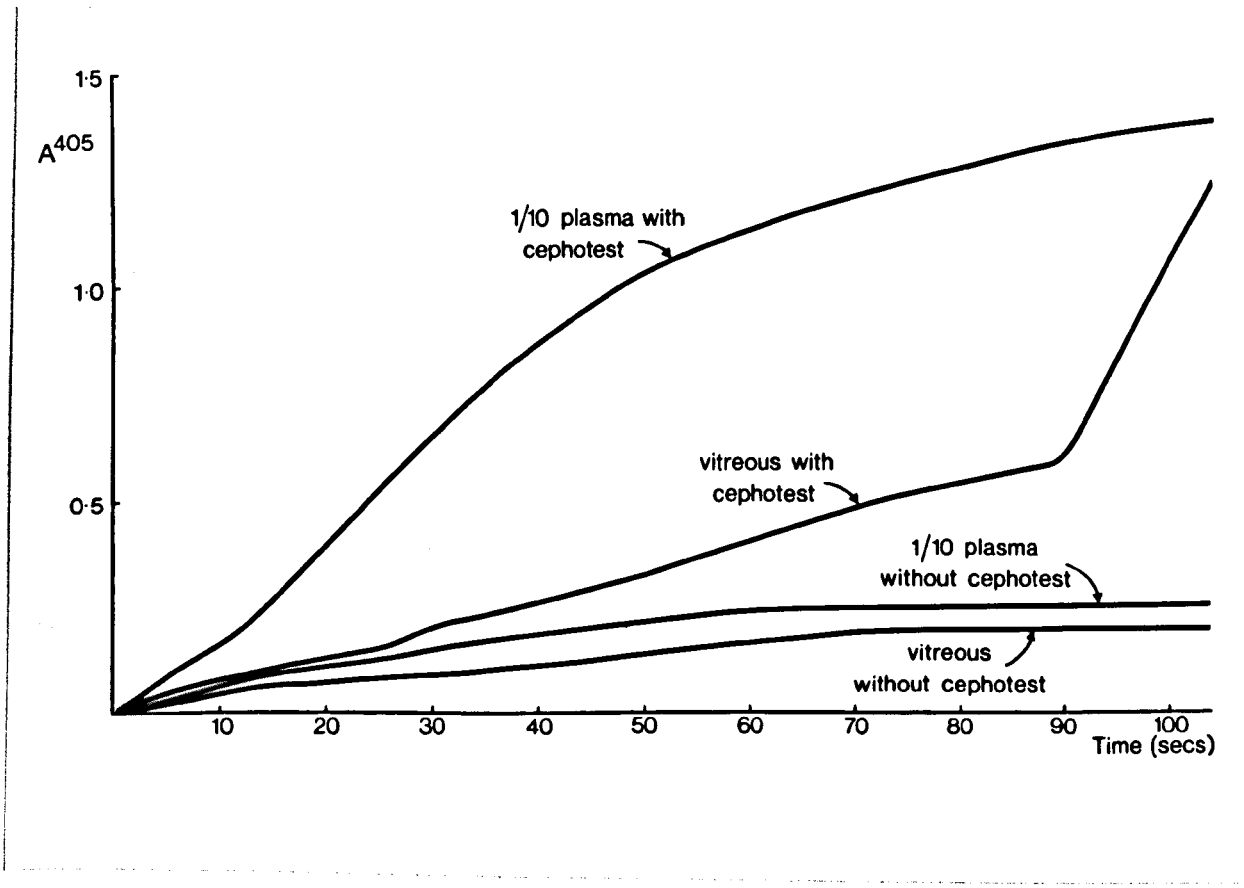


Figure 4.2 Time course of p-nitro-aniline (pNa) release from chromogenic substrate S2302 due to Hageman factor (Factor XII) activation in the presence of prekallikrein. Ordinate: absorbance at 405 nm of pNa. Abscissa: time (secs).

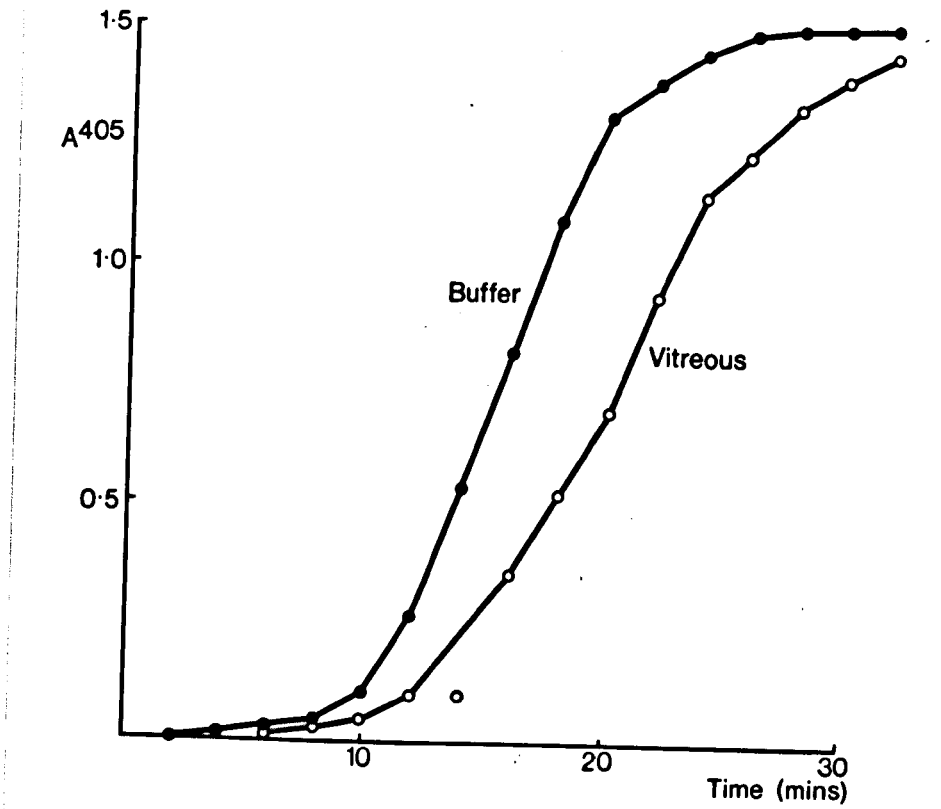


Figure 4.3 Time course of pNa release from chromogenic substrate S2222 in the presence of buffer and vitreous using a protein concentrate which contained Factors II, IX and X (Defix). Calcium ions were added to samples at time zero.

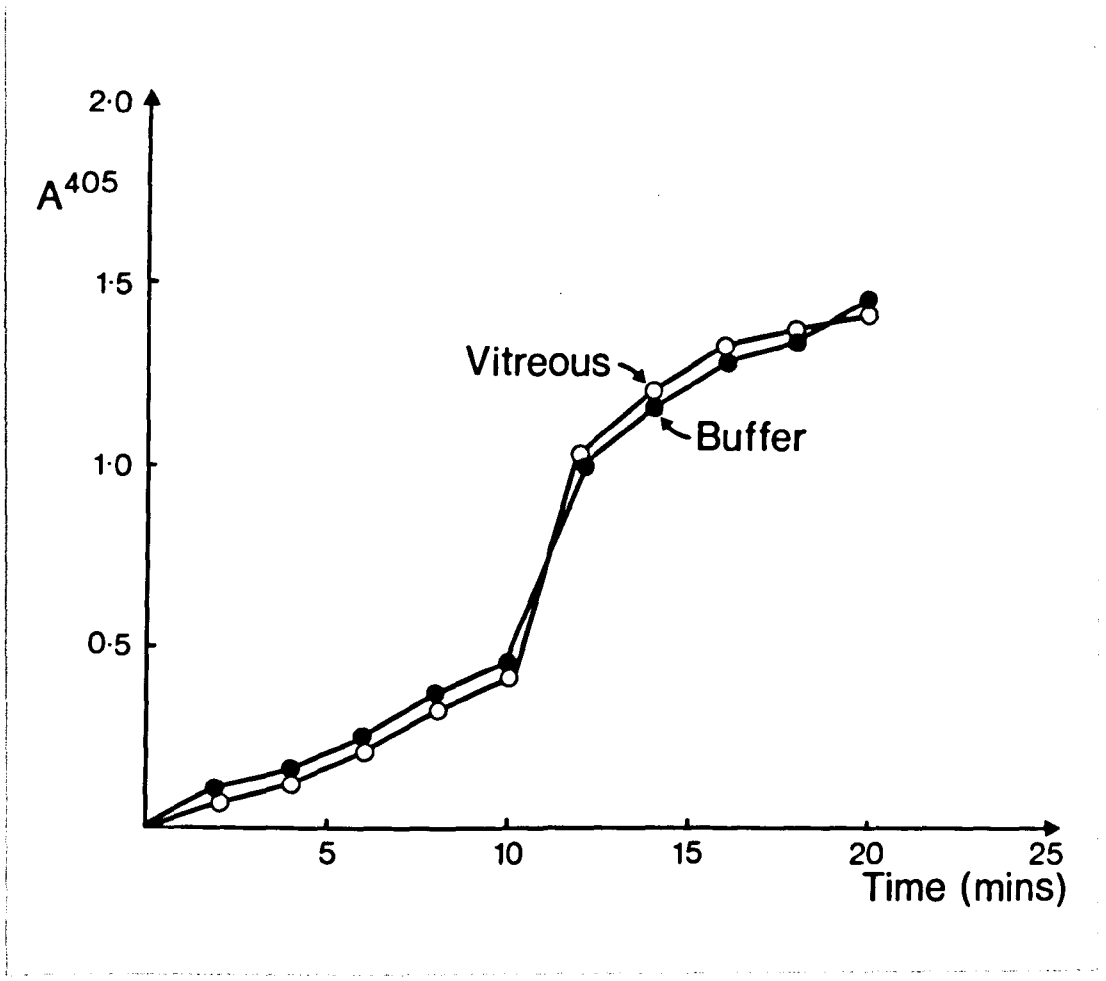


Figure 4.4 Time course of pNa release from chromogenic substrate S2222 in presence of vitreous and buffer, using a protein concentrate which contained Factors II, IX, X and VII, plus other unidentified activated components (FEIBA). Conditions as for Figure 4.3.

and negative (Tris-saline) controls were included. In one set of experiments (Table 4.5, column A), vitreous produced prolonged clotting times in the system when compared to the saline (negative) control, and thus vitreous appeared to be inhibitory to intrinsic prothrombin-converting activity in the preparations. In the second set (Table 4.5, column B) there was no difference between vitreous and the negative control, while in the third set (Table 4.5, column C), the vitreous caused slight shortening of the clotting time, suggesting the presence of minimal prothrombin-converting activity in the vitreous, equivalent to ecarin venom at an activity of 1 u/ml.

CLOTTING STUDIES IN THE PRESENCE OF VITREOUS GEL

It is known that the gel-forming component of the vitreous is its collagen fraction and that hyaluronic acid adds stability to the three dimensional collagen network (see Chapter 1). Previous studies have shown that isolated vitreous collagen has considerable platelet-aggregating ability[490]. The present experiments confirm and extend these observations.

Platelet aggregation

Addition of rabbit and bovine vitreous collagen to human platelet-rich plasma caused rapid platelet aggregation (Fig. 4.5, 4.6). The effect was dose-dependent, and could be inhibited by acetyl-salicylic acid. In addition, the normal lag phase seen with equine tail-tendon collagen suspension (Fig. 4.7) appeared to be shortened. This effect was not due to the sonication procedure used to suspend samples of vitreous collagen, since identical treatment of the equine tendon

TABLE 4.5

Assay of Prothrombin-converting Activity in Vitreous

Sample No.		Recalcification Times (secs) saline	vitreous	ecarin
A	1	505	1225	59*
	2	738	>3600	51*
B	3	>1800	>1800	1125/
	4	>1800	>1800	1140/
C	5	>3600	1294	1474/
	6	>3600	1495	1495/

System contained 0.1 ml each of purified prothrombin (one in five plasma activity), fibrinogen (2.5 mg/ml), Factor V (one in four plasma activity), and platelet substitute. Saline (negative control) and ecarin (positive control) were included.

* ecarin concentration = 16 u/ml.

/ ecarin concentration = 1.0 u/ml.

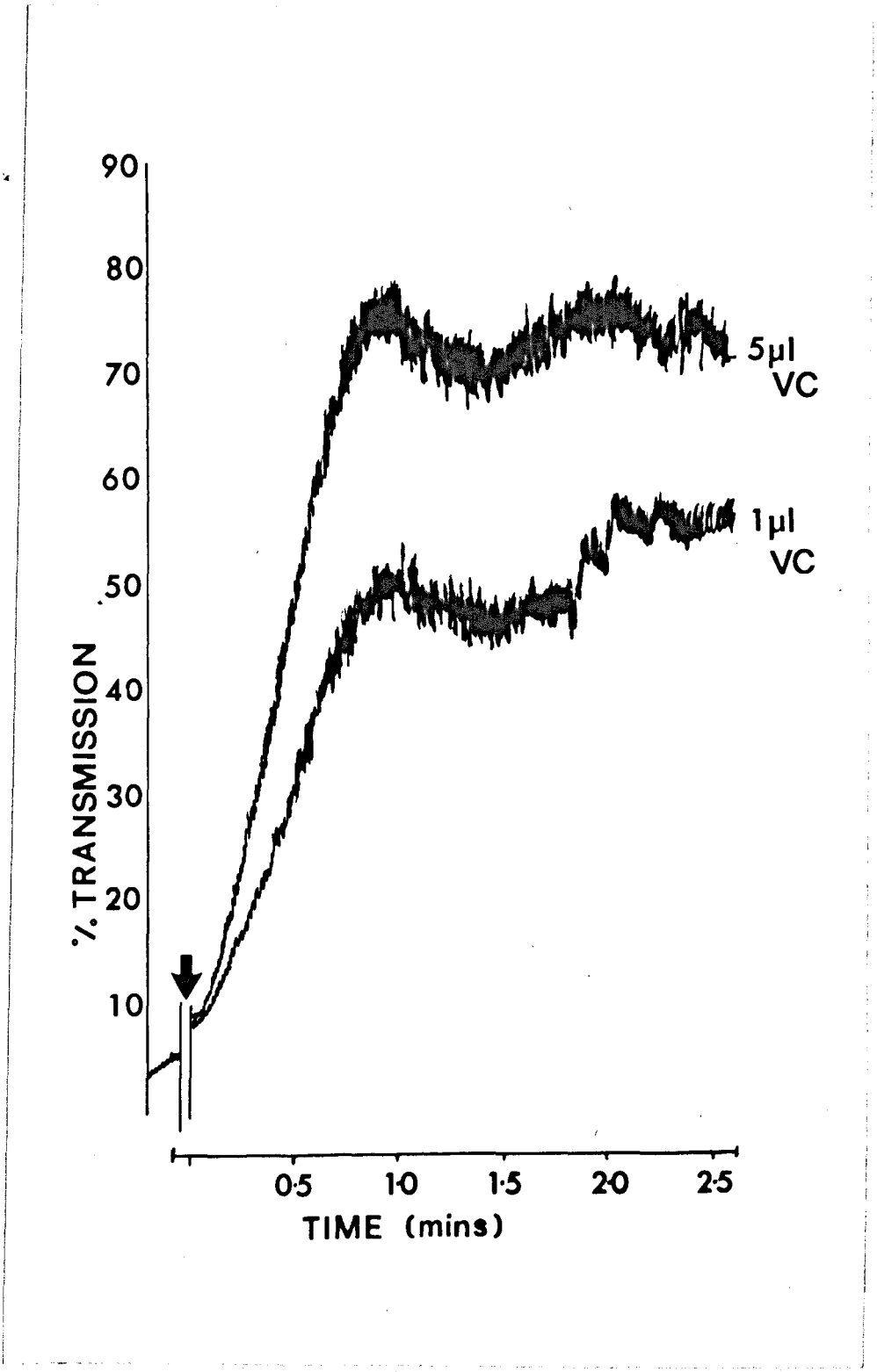


Figure 4.5 Optical density curve at 640nm for platelet aggregation in presence of rabbit vitreous collagen. Arrow denotes time of addition of vitreous collagen (VC). Lag phase is less than sixty seconds.

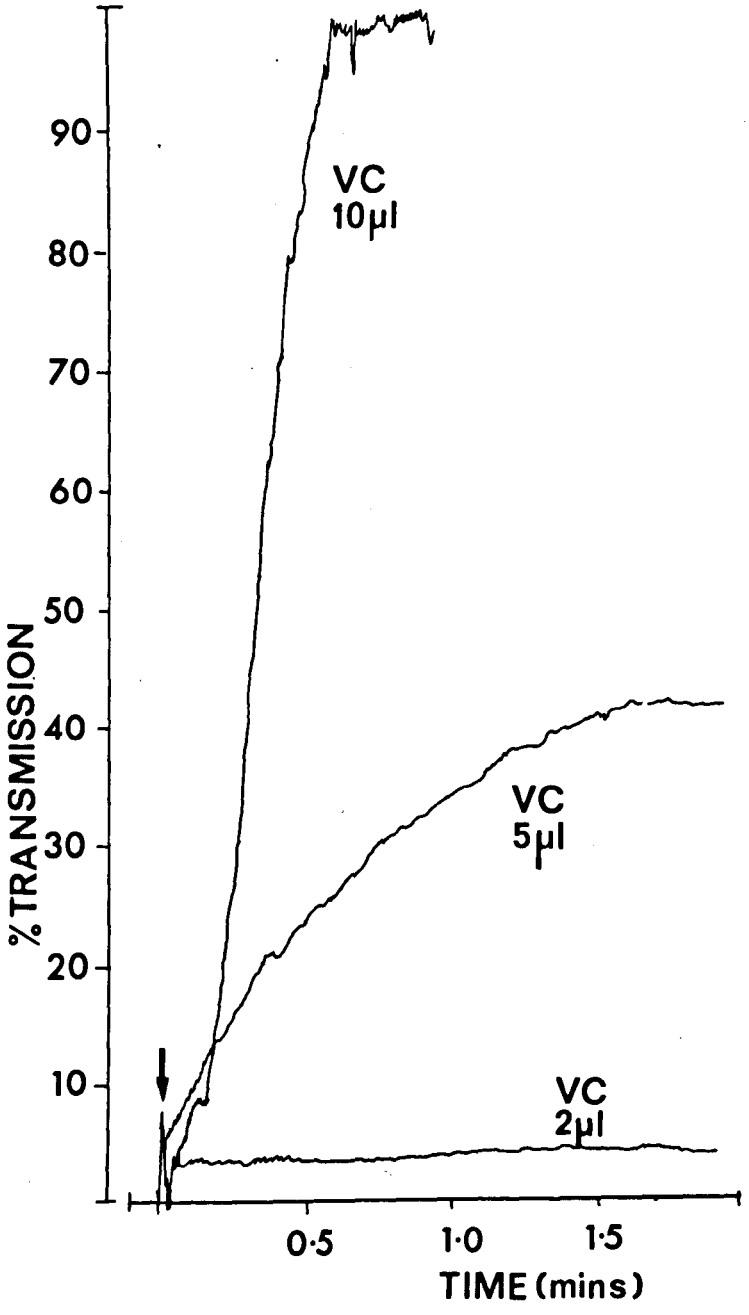


Figure 4.6 Optical density curve at 640 nm for platelet aggregation in presence of bovine vitreous collagen (VC). Note very short lag phase after addition of collagen (arrow). The aggregating effect of the collagen is dose-dependent.

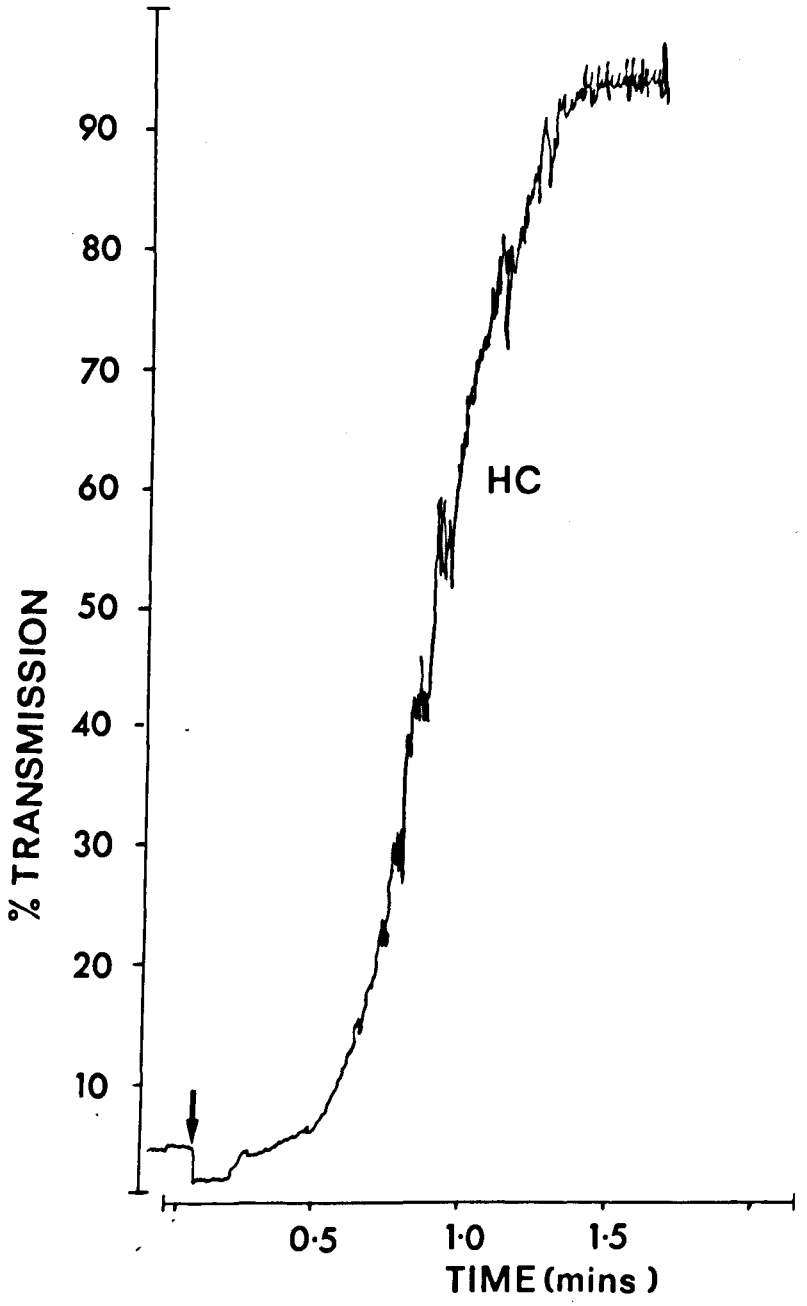


Figure 4.7 Optical density curve at 640 nm for platelet aggregation in presence of equine tail-tendon collagen (HC). Note typical lag phase of approximately sixty seconds before maximal response is achieved.

collagen suspension failed to shorten the lag phase. No platelet aggregation was observed with the supernatant obtained from the vitreous humour after ultracentrifugation. It was concluded that platelet aggregation observed in later studies in intact vitreous gels was due to its collagen component.

Fibrin formation in platelet aggregates within vitreous gels

Slow injection of citrated platelet-rich plasma into the centre of intact bovine vitreous gels resulted in a clot-like, loculated mass of plasma, which moved as a single structure in keeping with elastic changes induced in the gel by gentle agitation (Fig. 4.8). The same effect was observed with injections of whole blood, but not with platelet-poor plasma, serum or saline injections. These substances remained fluid and slowly tracked through the vitreous gel by gravity. This indicated that the 'globule forming' effect required the presence of platelets. Previous studies have suggested that injections of citrated platelet-rich plasma into the centre of the vitreous in vivo causes rapid platelet aggregation[490] and that this is a possible trigger for intrinsic system coagulation. In the present experiments, the possibility that a fibrin clot had occurred in the globules of citrated platelet-rich plasma was investigated by searching for fibrin within the vitreous gels.

(a) Histology

Histological staining of platelet-rich plasma 'globules' in vitreous gels with Martius scarlet blue (MSB) showed considerable positive staining reaction in platelet masses adherent to vitreous collagen (Fig. 4.9c). The clot-like mass in the vitreous was composed of an outer layer of

Figure 4.8 Macrophotograph of a plasma 'globule' within a bovine vitreous gel. This clot-like structure was obtained by slow injection of citrated platelet-rich plasma into the centre of the vitreous gel through a fine needle, in the absence of added calcium ions.

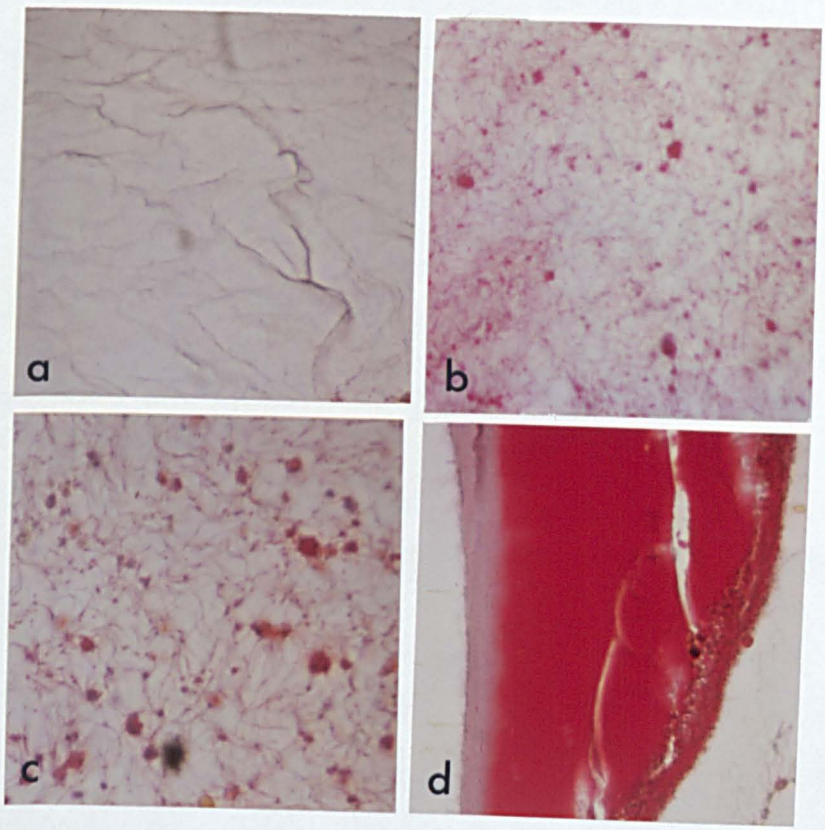


Figure 4.9 Histology of citrated platelet-rich plasma 'globules' with bovine vitreous gels (for explanation, see text) (MSB stain).

(a) control normal vitreous collagen. x 400

(b) control thrombin-clotted platelet-rich plasma clot, showing typical reticular pattern of fibril. x 400

(c) Positive (red) staining of platelets adherent to vitreous collagen. x 400

(d) Section through plasma-vitreous 'globule'. Intense, granular staining within centre of globule. No typical clotted fibrin shown. x 400

platelets and a central core of granular material, which also showed strongly positive staining with MSB (Fig. 4.9d). However, the typical reticular pattern of a fibrin clot, seen in control sections of a thrombin-induced platelet-rich plasma clot (Fig. 4.9b) was not detected. The absence of fibrin strands within the vitreous mass suggested that a true fibrin clot had not occurred. Positive MSB staining reaction of granular material within the vitreous gel may have occurred as a result of dye uptake by non-clotted fibrinogen which had become loculated within the gel and was precipitated during the fixation process. The possibility that the granular material represented an atypical form of clotted fibrin could not be excluded.

(b) S.D.S. polyacrylamide gel electrophoresis

Vitreous gels which had been injected with 0.5 ml citrated platelet-rich plasma were disrupted by ultracentrifugation (see Chapter 3) and the pellet was solubilised with S.D.S. and β-mercaptoethanol after thorough washing in 0.9% saline. No fibrinogen or fibrin chains were detected in the samples by S.D.S. polyacrylamide gel electrophoresis (Fig. 4.10). A number of other protein bands, whose mobility on gel electrophoresis corresponded more closely with intrinsic platelet proteins, were detected. These results indicated that fibrin formation did not occur in vitreous gels in which citrated platelet-rich plasma had been injected.

Effect of platelet aggregation by vitreous collagen on plasma clotting times

The absence of fibrin formation in platelet aggregates in vitreous gels indicated that in this experimental system, platelet activation was

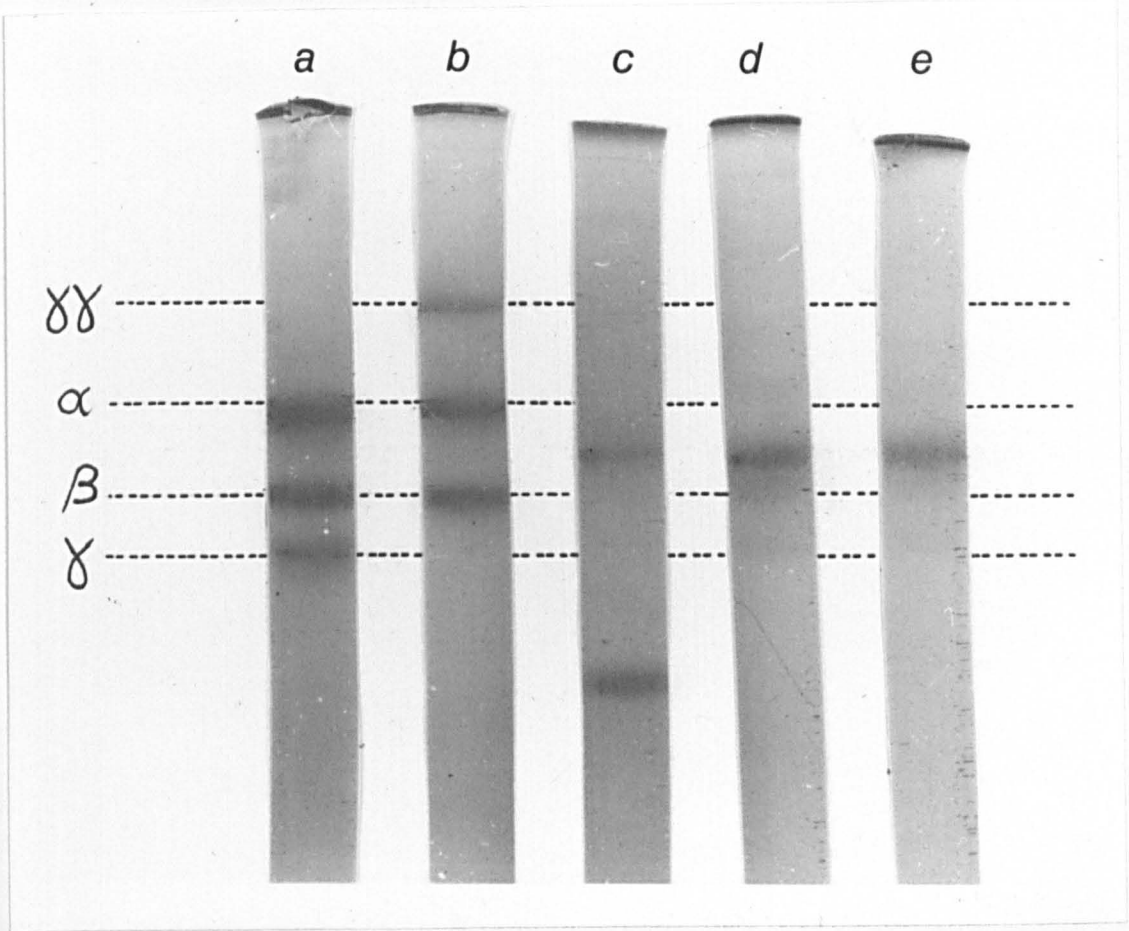


Figure 4.10 Detection of fibrin by S.D.S. polyacrylamide gel electrophoresis in platelet-rich plasma 'globules' within bovine vitreous gels (for explanation, see text).

(a) Control non-cross-linked human fibrin

(b) Control partially cross-linked human fibrin from washed plasma clot

(c) Control protein bands from washed non-aggregated human platelets.

(d) and (e) protein bands obtained from platelet-rich plasma globules in vitreous gels. α, β, γ and $\gamma\gamma$ dimer refer to constituent fibrin chains (see Chapter 2). Note absence of these chains in samples from plasma globules in vitreous, indicating that fibrin conversion had not occurred.

associated with any thrombin-like activity. However, the release of other platelet procoagulant activity may have been masked by the absence of free calcium ions. The present experiment was therefore undertaken to explore this possibility. The recalcification times of platelet-rich plasma and platelet-poor plasma were compared after incubation of 0.1 ml of each plasma with 0.1 ml bovine vitreous collagen suspension for 2 minutes at 37°C. Control samples were incubated with Tris-saline buffer under identical conditions. In addition, samples of platelet-poor plasma in the presence of platelet substitute (platelin) were clotted in the same manner to exclude any effect due to non-specific phospholipid activity as distinct from platelet aggregation. The results are shown in Table 4.6. The recalcification time of platelet-poor plasma was slightly shortened in the presence of vitreous collagen. The effect was similar in the presence or absence of platelet substitute. The recalcification time of platelet-rich plasma was further shortened in the presence of vitreous collagen, at a dose sufficient to cause platelet aggregation. This suggested that procoagulant activity was released from the platelets after aggregation and that the effect was not due to platelet phospholipid alone.

DISCUSSION

The vitreous contains three classes of macromolecules, namely collagen, hyaluronic acid and traces of soluble proteins, mainly glycoproteins, which are in part derived from the blood (see Chapter 1). In the present experiments, an attempt was made to define the role of each of these constituents on coagulation mechanisms in the vitreous. In the first series of experiments, untreated crude vitreous and collagen-free dialysed vitreous humour were tested for their effect on plasma

TABLE 4.6 Effect of Bovine Vitreous Collagen-induced Platelet Aggregation on Recalcification Time of Human Plasma

Sample	Recalcification Times (secs)					
	PRP* + Buffer	PRP + Vitreous collagen	PPP/ + Buffer	PPP + Vitreous collagen	PPP + Platelet substitute + Buffer	PPP + Platelet substitute + Vitreous collagen
1	220	173	332	301	210	198
2	221	185	329	325	227	195
3	205	190	341	327	225	193
4	230	181	347	308	221	191
5	229	164	347	321	220	191
6	223	179	317	320	217	189
Mean	221.3	178.7	335	317	220	192
S.D.	9.0	9.2	11.8	10.3	6.1	3.2
S.E.M.	3.7	3.7	4.8	4.2	2.5	1.3
P	<.0001		.0157		<.0001	
Mean difference in recalcification time	42.7±6.7		19.0±6.3		27.2±3.1	

* PRP = platelet-rich plasma

/ PPP = platelet-poor plasma

recalcification times using cell-free plasma. The vitreous had no effect on clotting of surface-activated normal plasma in glass tubes, but human vitreous caused some shortening of plasma clotting time when plastic tubes were used, suggesting the presence of Factor XII activity (Hageman factor) in the vitreous. This possibility gained support from experiments showing a significant shortening of Factor XII-deficient plasma by collagen-free dialysed bovine vitreous (Table 4.3). Furthermore, trace amounts of a Hageman-factor-like moiety were detected in vitreous samples which were tested for prekallikrein-converting activity (Fig. 4.2). These results confirmed the work of Regnault and Larrieu[421] who noted similar trace amounts of Factor XII activity in human vitreous. The source of this activity in vitreous was more difficult to define. Since Hageman factor is a glycoprotein of molecular weight 80,000 daltons (see Chapter 2), it is conceivable that traces of this protein could diffuse through the blood-vitreous barrier by a process of ultrafiltration[282] and form part of the plasma-derived soluble glycoproteins of the vitreous (see Chapter 1). The lack of effect of pure hyaluronic acid, in contrast to the marked effect of vitreous, on Factor XII-deficient plasma would suggest that Hageman factor-like activity in the vitreous resides within its soluble glycoprotein component (Table 4.3). However, since only trace amounts of Hageman factor activity were detected (Fig. 4.1), its physiological role is uncertain.

Apart from any intrinsic Hageman factor activity, the vitreous itself may activate plasma Hageman factor, during episodes of bleeding, by virtue of its high content of hyaluronic acid. Although this macromolecule had little effect on the clotting times of surface-activated normal or Factor XII-deficient plasma, a significant reduction in the recalcification time of non-activated plasma, in the presence of vitreous and hyaluronic acid was observed (Table 4.4, Fig. 4.1). Hyaluronic acid shows structural

similarity with polymeric polysaccharides such as heparin and chondroitin sulphate which, in common with other negatively-charged molecules, have been shown to activate Hageman factor and generate kinin release[345] (see Chapter 2). Hageman factor activation in the vitreous would provide a mechanism for clotting via the intrinsic pathway.

There appeared to be few other coagulation factor activities in the vitreous. Fibrinogen and thrombin activity were not detected, nor was there any significant Factor II or prothrombin-converting activity (Tables 4.3, 4.5). A search for Factor Xa activity also showed negative results (Fig. 4.3). Indeed, vitreous appeared to delay the generation of Factor Xa activity in coagulation factor concentrates (Fig. 4.3). These results contrast with those of Regnault and Larrieu[421], who considered vitreous to contain Factor Xa activity on the basis of clotting experiments using purified prothrombin. However, the possibility of minimal contamination with Factor X in their samples of prothrombin, which were used at 10 fold activity of normal plasma, was not excluded. Moreoever, the ionic composition of the vitreous samples used in their study was not controlled.

The prolongation of the recalcification time of Factor VII-deficient plasma by dialysed bovine vitreous suggested that Factor VII was a requirement for intravitreal clotting. This would indicate a predominant role for extrinsic system clotting in the vitreous in spite of low levels of tissue thromboplastin[382]. However, vitreous had no effect on the generation of Factor VIIa when compared with buffer (Fig. 4.4). An alternative explanation for the delay in Factor VII-deficient plasma clotting times may be that the vitreous contained an inhibitor of intrinsic system coagulation since, using this plasma, extrinsic clotting was effectively blocked. Further studies have shown that traces of inhibitor (α_1 antitrypsin) are present in normal vitreous

(see Chapter 6). There is, therefore, little direct evidence that clotting in the vitreous occurs via the extrinsic coagulation mechanism, as it does in other tissues. On the contrary, the low levels of tissue thromboplastin in the vitreous suggest that this is not the dominant mechanism, although clearly it may contribute to the clotting process in a minor fashion.

The role of platelets in fibrin formation within the vitreous was investigated. Previous authors have reported that vitreous collagen possesses considerable platelet aggregating ability[490], and they suggested that clotting in the vitreous was triggered by platelet procoagulant activity. In the present work, both rabbit vitreous collagen which is Type IV (see Chapter 1) and bovine vitreous collagen (Type II) demonstrated a pronounced and dose-dependent ability to cause platelet aggregation, when prepared as a suspension by ultrasonication (Figs. 4.5, 4.6). In addition, the normal lag phase found with similarly-treated equine tendon collagen (Fig. 4.7) was shortened. In the studies reported by Swann et al[490], who used mainly guanidine-HCl extracts of bovine vitreous collagen, the platelet aggregating ability of vitreous collagen was an all-or-none effect and the initial lag phase was considerably longer. Current views of the collagen-platelet interaction suggest that the lag phase in platelet aggregation by collagen is the result of a preliminary step whereby soluble tropocollagen molecules co-aggregate to form microfibrils, which are considered to be a pre-requisite for collagen-induced platelet aggregation[30]. The shortened lag phase observed in the present study may therefore be explained. Since vitreous occurs as fine fibrils or microfibrils in its natural state (Chapter 1), a suspension of this material may have required no further realignment to induce platelet aggregation.

Although vitreous collagen caused rapid platelet aggregation, evidence for fibrin formation, solely as a result of this response,

was not found. Injection of citrated platelet-rich plasma into intact vitreous gel resulted in a clot-like mass which, on histological examination, lacked the characteristics of a typical reticular fibrin clot (Fig. 4.9). Furthermore, analysis of the solid material obtained from the vitreous on S.D.S. polyacrylamide gel electrophoresis failed to reveal the typical profile of fibrin (ogen) chains (Fig. 4.10). These results indicated that platelet aggregation by vitreous collagen was not associated with any direct thrombin-like activity. However, platelet procoagulant activity is well recognised (see Chapter 2), and requires the presence of free calcium ions. It is probable, therefore, that platelet aggregation by vitreous collagen is coupled with release of procoagulant activity. Evidence to support this concept is shown in Table 4.6, where the recalcification time of platelet-rich plasma after incubation with vitreous collagen was significantly shorter than the recalcification times of platelet-poor plasma or platelet-poor plasma plus platelet substitute after similar incubation with vitreous collagen. These results indicate that, although platelets have no thrombin-like activity, clotting is accelerated in the presence of intact platelets and vitreous collagen, and is probably due to the release of platelet procoagulant activity after collagen-induced platelet aggregation.

CONCLUSION

Blood coagulation within the vitreous may occur by several mechanisms. Unlike other tissues, tissue thromboplastic activity of the vitreous is low and therefore clotting via the extrinsic system may not be the predominant mechanism. On the contrary, the effects of vitreous and hyaluronic acid on non-activated plasma suggest that plasma

Hageman factor may be activated by the vitreous, thereby initiating clotting via the intrinsic system. In addition, the vitreous contains traces of endogenous Hageman factor-like activity, although its role in intravitreal clotting is somewhat obscure.

Clotting in the vitreous may also be accelerated, if not initiated, by the considerable platelet-aggregating ability of vitreous collagen which, almost certainly, is associated with the release of platelet-procoagulant activity. In addition, experiments with citrated platelet-rich plasma in intact vitreous gels indicate that plasma may be trapped or loculated within the vitreous, thereby producing conditions which would favour fibrin conversion by one or all of the above mechanisms.

PART 3

ROLE OF FIBRINOLYSIS IN THE RESOLUTION OF VITREOUS BLOOD CLOTS

CHAPTER 5

REVIEW OF THE PHYSIOLOGY OF FIBRINOLYSIS

INTRODUCTION

The conversion of circulating plasma fibrinogen to insoluble fibrin during the last stages of blood coagulation represents the main defence mechanism in haemostatic control. In addition to forming the major protein component of blood clots, fibrin is deposited in substantial quantities within fresh wounds and may be important in providing a scaffold for the invasion of cells[41,343,447]. During such processes as wound healing or recanalisation of vascular thrombi, fibrin deposits are gradually dissolved. Over thirty years ago, it was recognised that fibrin dissolution was achieved by an enzyme which was present in normal plasma as an inactive precursor, plasminogen, but which could be converted to the active proteinase, plasmin, by a streptococcal factor[249]. Since then, intensive investigation of the fibrinolytic process has shown that several other components participate in this system, and the recently recognised interactions of fibrinolytic factors with the coagulation process, the complement system and the kinin-forming mechanism in inflammation, has revealed the exquisite complexity of these homeostatic systems[245].

As Astrup[11] has emphasised, although several enzymes are capable of degrading fibrin, "fibrinolysis" is a specific term which describes the limited proteolysis of fibrin to produce liquefaction of a fibrin clot by splitting only a few peptide bonds. It is generally accepted that fibrin dissolution in vivo occurs predominantly via the plasminogen-plasmin system; the components of this system are illustrated in Figure 5.1. It can be seen that there is a general similarity to the coagulation scheme in that there are intrinsic and extrinsic means of achieving fibrinolysis. However, differences exist between the extrinsic and intrinsic fibrinolytic systems in the exact mechanism

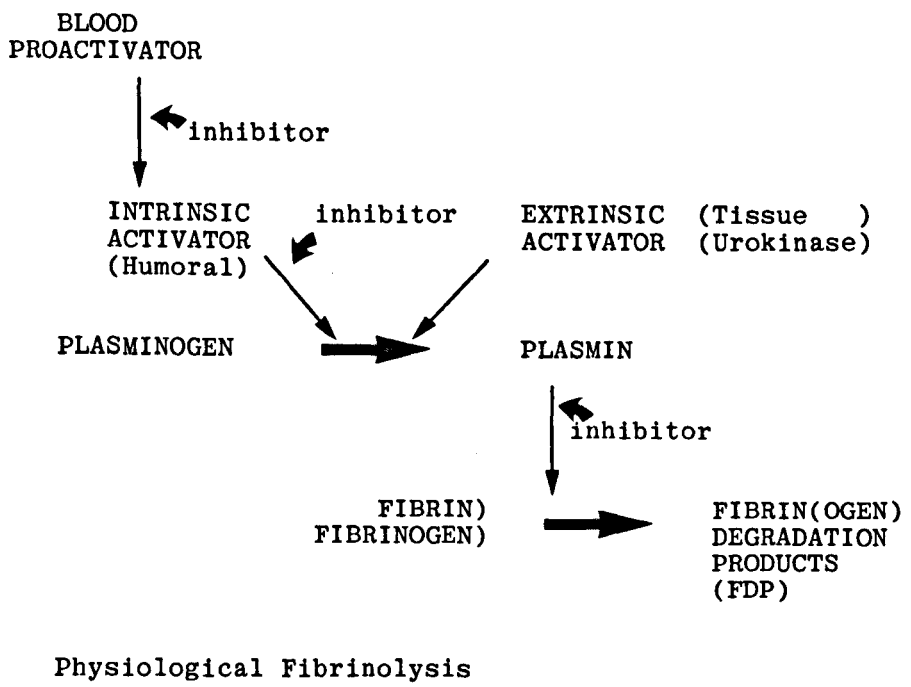


Figure 5.1 Physiological Fibrin(ogen)olysis

of fibrin degradation which depends on a number of factors such as the affinity of the various activators, plasmin or both for fibrin and fibrinogen molecules. These are discussed below.

THE PLASMINOGEN-PLASMIN SYSTEM

The four components of this system are plasminogen, plasmin, plasminogen activators and inhibitors.

PLASMINOGEN

The introduction in 1970 of a method for the purification of plasminogen from human plasma by lysine-sepharose affinity chromatography has led to a much greater understanding of the chemistry of this molecule[120]. Human plasminogen exists as a single-chain β-globulin of about 90,000 daltons[477,488], but several molecular forms may occur. Studies of the conversion of plasminogen to plasmin have revealed that at least two forms of plasminogen are generated, differing in the NH_2-terminal portion of the molecule. Native plasminogen occurs with glutamic acid as its amino-terminal (Glu-plasminogen), while limited proteolysis of Glu-plasminogen with urokinase yields Lys-plasminogen, which contains lysine at its amino-terminal. Lys-plasminogen has a shorter half-life[102], is more rapidly activated[536] and is more readily adsorbed to fibrin than Glu-plasminogen. Associated with the conversion of Glu-plasminogen to Lys-plasminogen is a conformational change in the shape of the molecule[472] which is also seen when e-aminocaproic acid is bound to the molecule[472]. This change in molecular shape has been invoked as an explanation for the biphasic pattern of fibrinolysis-inhibition

of urokinase by e-aminocaproic acid[507]. The primary structure of human plasminogen has recently been elucidated in some detail and the molecule consists structurally and functionally of three parts which are proteolytically cleaved when activation occurs[305]. The part corresponding to the heavy chain of plasmin contains five triple disulphide loops (known as "Kringle" structures) which not only have internal homology, but are also homologous to certain regions in the prothrombin molecule[479] (see Chapter 2).

PLASMIN

Limited proteolytic cleavage of Lys-plasminogen yields the serine protease, plasmin. There has been some disagreement concerning the sequence of reactions involved in this process, mainly because of apparent differences in the mode of action of plasminogen activators. Cleavage of plasminogen with urokinase occurs at an arginine-valine bond in the carboxy-terminal portion of the molecule[440], This results in a double chain plasmin molecule, linked by a single disulphide bridge (Fig. 5.2). The active site of plasmin occurs on the light chain. Streptokinase activation of plasminogen also produces a double chain plasmin molecule, but unlike urokinase, it does not activate plasminogen directly (vide infra). Although it was previously thought that conversion of Glu-plasminogen to Lys-plasminogen with release of a 7,000 dalton peptide[433] (pre-activation peptide, Fig. 5.2) was a pre-requisite for plasmin production, it has been shown that this is not the case[489]. Recent evidence suggests that plasmin production can proceed by two pathways, the major one involving the conversion of Glu-plasminogen to Lys-plasminogen by catalytic amounts of plasmin (Fig. 5.3)[523].

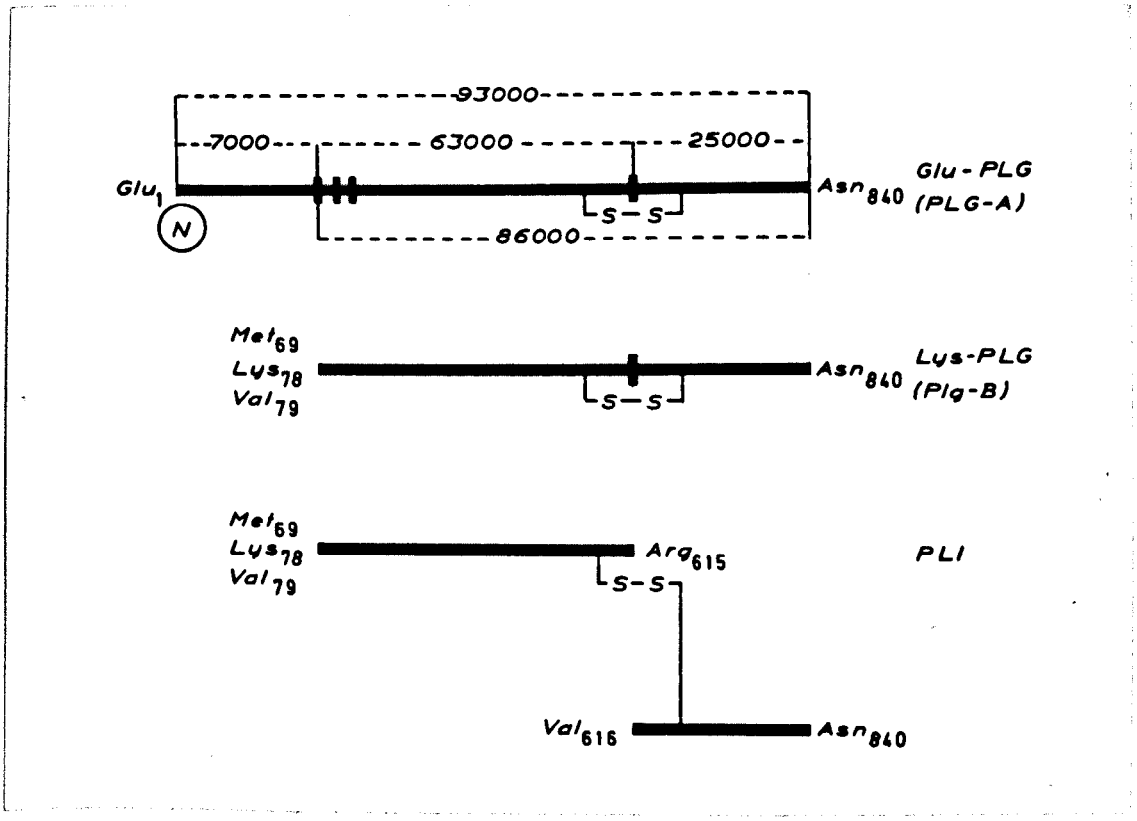


Figure 5.2 Schematic representation of the chain composition of Glu-plasminogen, Lys-plasminogen and plasmin (Wallen[535]). Figures refer to the molecular weights of various plasminogen fractions. Release of a 7,000 dalton peptide (pre-activation peptide) occurs during conversion of Glu-plasminogen (Glu-PLG) to Lys-plasminogen (Lys-PLG). PLI, plasmin: -S-S, disulphide bond.

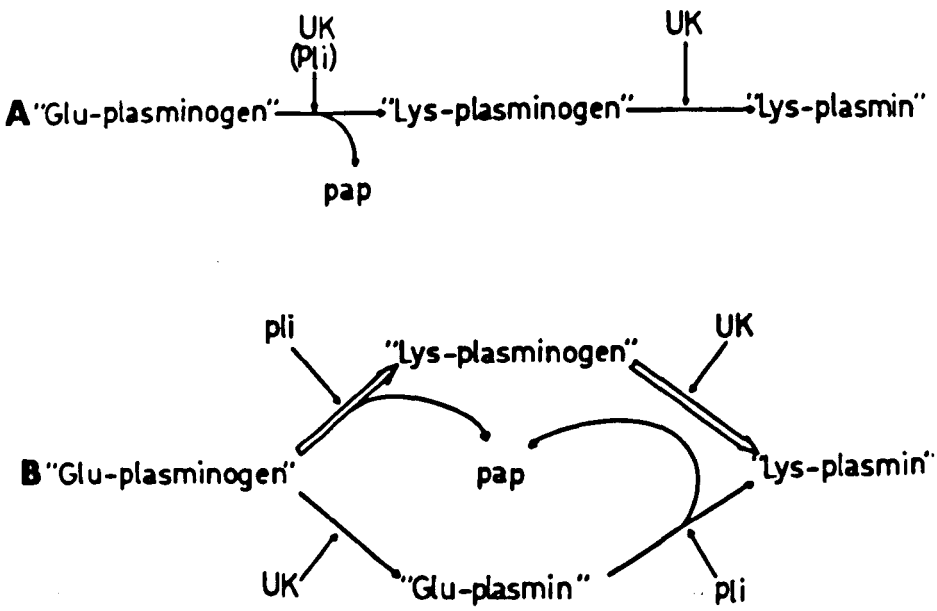


Figure 5.3 Two-pathway model for activation of plasminogen, pli- catalytic amounts of plasmin: UK - urokinase; p.a.p. - preactivation peptide. (Wallen,[535]).

PLASMINOGEN ACTIVATOR

The active principle responsible for the conversion of plasminogen to plasmin is known as plasminogen activator. Studies of the mechanism of plasminogen activator interaction with plasminogen have been complicated by the heterogeneity of activators, which may show both functional and immunological non-identity, depending on the source. Several plasminogen activators have been described such as tissue activator, plasma activator, vascular endothelial activator, Factor XII-dependent activator, cellular activator and urokinase. Bacteria also synthesise activator, of which the most studied is streptokinase. Some of these activators are described below.

PLASMA PLASMINOGEN ACTIVATOR

Normal plasma shows a limited degree of spontaneous activator activity, which is labile and shows considerable inter-species variability[361]. Increased activity has been observed after a variety of stimuli such as venous occlusion, strenous exercise, emotional stress, surgical operations and administration of vasoactive amines (for review see reference 10), and plasma activator levels may be altered by therapy in systemic diseases such as diabetes mellitus[137]. It has been suggested that release of activator activity in the blood is under humoral and possibly hypothalamic control[77].

The origin of plasma plasminogen activator is the source of considerable debate. At present, at least three mechanisms appear to contribute to plasma activator activity; the vascular endothelium, the Hageman-factor activatable mechanism, and the "peptone effect".

Vascular Endothelial activator

Indirect evidence, based on a standardised venous occlusion test which produces a rise in fibrinolytic activity in the blood, suggests that activator may be released from the vascular endothelium[534]. It has been observed, however, that endothelial activator is strongly cell-bound and stable[13], unlike plasma activator. In this respect, endothelial activator closely resembles tissue activator (see below). Conversely, saline extracts of tissues are not entirely stable[2], and it is possible that endothelial activator also has a labile component which may correspond to plasma activator activity.

Hageman-factor dependent pathways for plasmin production

Activation of Hageman factor by negatively-charged surfaces via high molecular weight kininogen (see Chapter 2) induces fibrinolytic activity in plasma[246]. The complex inter-relationships between coagulation and kinin-formation are further emphasised by the observation that prekallikrein, which is cleaved by activated Hageman factor, also acts as a plasminogen proactivator since its product, kallikrein, can convert plasminogen directly to plasmin[247]. The physiological role of this system is not clear, but reduced fibrinolytic activity has been detected in Hageman factor deficient plasma[549].

The "Peptone Effect"

Peptone added to guinea pig serum produces fibrinolytic activity in the euglobulin (inhibitor-free) fraction[14,515] of blood plasma which is independent of Hageman factor. This effect was found to be a general property of acid polysaccharides such as heparin, dextran sulphate, cellulose trisulphate and hyaluronic acid[368]. Several investigations

suggest that the "peptone effect" depends upon the presence of a humoral plasminogen proactivator in blood which is activated to a large extent on the removal of inhibitors by the polyelectrolytes[14].

Other mechanisms of fibrin digestion by blood factors, independent of plasminogen activator activity, have been proposed. These include a second Hageman-factor independent mechanism, possibly involving C_3[461], a further separate mechanism which requires the participation of platelets, IgM, C_3 and C_4[501], and finally, fibrin removal by proteinases from white blood cells[48]. It has been suggested that such mechanisms may have equal, if not greater significance in the digestion of fibrin clots by blood or blood products[340].

TISSUE ACTIVATOR

Plasminogen activator activity has been demonstrated in almost all tissues of the body, either in saline extracts of tissues[2] or by Todd's fibrin-slide histochemical technique[509]. Tissue activator is highly stable (although a small component of the total activity is labile, see above), and strongly resists saline extraction. As a result, molar potassium thiocyanate is generally required for its quantitative extraction, and more recent studies using this technique have shown differences in activator activity among various tissues[9,79]. Highly purified tissue activator has been prepared from pig heart[18,96] and pregnant hog ovaries[258]. Molecular weight studies of plasminogen activators from these and other sources indicate a range of 57,000 to 80,000[6,7,390]. The lower value corresponds more closely to similar studies of molecular weight for plasma activator[371]. Tissue activator activity is high in vascularised connective tissues, especially in healing wounds[392]. Activity is highest in venous endothelium, but is

low in arterial endothelium while capillaries are usually inactive[510]. However, capillary buds and areas of neovascularisation show high activity, which subsequently disappears after scar formation[274]. The relationship between fibrinolytic activity and degree of vascularity of any one tissue has been emphasised by Astrup[9]. Although venous endothelial cells are considered to be the source of most of this activity, there is considerable variation in the distribution of active sites[178]. Indeed, cellular studies suggest that there may be a cycle of activator release. Proliferating cells are generally inactive, but during maturation and differentiation intracellular synthesis of the protease takes place, which is then released as the life cycle of the cell approaches the stage of involution and degeneration[12]. Fractionation studies of cell homogenates indicate that plasminogen activator activity is located in the microsomal and lysosomal fractions[277] of the cell.

UROKINASE

Urokinase (UK) is a β-globulin with a single polypeptide chain (molecular weight 54,000) which is found in human urine and has plasminogen activator activity[292]. Although previously considered to be an excretion product of activator produced elsewhere in the body, urokinase is now believed to be secreted by the mucosal cells lining the kidney ductules[47]. In addition, UK appears to differ chemically from tissue activator[258] and to have a considerably lower affinity for fibrin[506]. The physiological role for the uniqueness of the many plasminogen activators in various tissues and fluids is not yet clear, but identity can occasionally be established between activators, for instance, between urokinase and a plasminogen activator from a human ovarian carcinoma cell line[8]. Urokinase activates plasminogen directly (see above).

STREPTOKINASE

Of the many fibrinolytic enzymes produced by bacteria, streptokinase (SK) has been most studied. Streptokinase is an α-globulin of molecular weight 47,000, and is produced by β-haemolytic streptococci[210]. It activates plasminogen by a two-step process, previously thought to require the presence of a blood proactivator, but now known to involve complex formation between SK and plasminogen[420]. SK-plasminogen undergoes a transition to SK-plasmin[19] which is capable of converting plasminogen to plasmin[456]. It has been proposed that free plasmin can be produced by excess quantities of plasminogen merely by exchange with the SK-plasmin complex[263]. Recent studies have discounted this mechanism, and adhere to the generally held view that the SK-plasmin complex cleaves the plasminogen molecule in the same way as other activators[185]. However, the full sequence of events in plasminogen activation by SK is sufficiently different from other activators that it can be regarded as non-physiological.

OTHER SOURCES OF PLASMINOGEN ACTIVATOR

In addition to its association with vascular endothelium, plasminogen activator activity has been detected in several cell types including glandular epithelium[353], corneal epithelium[381], liver tissue[353] and embryonic kidney cell lines[378] under specific conditions. Furthermore, leukocytes contain enzymes which can digest fibrin by both plasmin-mediated and non-plasmin-mediated mechanisms[192,402]. Both pathways can be distinguished by the type of fibrinolysis products which are released[48], and it has been suggested that the non-plasmin-mediated pathway may be more important in pathological conditions[402]. In

addition to leukocytes, macrophages in culture can secrete large quantities of plasminogen activator into the extracellular fluid when the cells are appropriately stimulated[516], and this enzyme secretion can be modulated by anti-inflammatory steroids, mitotic inhibitors and cyclic nucleotides[521]. The secretion of proteolytic enzymes in this manner has been proposed as a major factor in the migration of cells into areas of inflammation. In addition, the enhanced fibrinolytic activity of malignant cells both in vivo[495] and in vitro[437] has stimulated considerable interest in the possible relationship between proteolytic activity and tumour spread[425].

INHIBITORS OF FIBRINOLYSIS

NATURALLY OCCURRING INHIBITORS

One of the major problems which has hindered the investigation of circulating plasminogen activators is the high content of naturally occurring inhibitors of fibrinolysis in blood. Two main types of inhibitor occur, namely inhibitors of plasminogen activation (anti-activators) and inhibitors of plasmin (antiplasmins).

Antiactivators

Antiactivators have been identified and separated from blood fractions[40]; their electrophoretic mobility corresponds to that of α_2 globulins with molecular weights of 75,000-80,000 and they can inhibit a variety of activators, which may suggest that their action is on the plasminogen molecule rather than the activator. Although specifically designed assays are available which show that these

proteins do inhibit activator[283], the existence of specific anti-activators with no effect on plasmin has not been clearly demonstrated.

Antiplasmins

Within the group of antiplasmins are the broad-spectrum plasma protease inhibitors, i.e. α_2 macroglobulin, α_1 antitrypsin, anti-thrombin III-heparin complex, C_1 inactivator and inter-α-trypsin inhibitor. These have been briefly reviewed in Section 1. Although it has been generally considered that most of the antiplasmin activity is contained in α_2 macroglobulin (reversible, rapid reaction with plasmin) and α_1 antitrypsin (irreversible, slow reaction with plasmin)[108], a further fast-reacting antiplasmin has recently been reported[132] and characterised[100,561]. It has been suggested that plasmin is inhibited by complex formation between the light chain of plasmin and the peptide-cleaved inhibitor[100]. Most recently, it has been shown that plasmin and antiplasmin react in a biphasic manner, the first of which is reversible[562]. The data suggest that circulating plasmin is rapidly neutralised, but fibrin-bound plasmin is not.

Antiplasmins are also detectable in cells and tissues such as platelets[240] and granulocytes[567] and arterial walls[145].

SYNTHETIC INHIBITORS OF FIBRINOLYSIS

Several synthetic agents can inhibit fibrinolysis either as anti-activators or, in higher concentration, as antiplasmins. These include e-aminocaproic acid (EACA)[327] and tranexamic acid (4-aminomethylcyclo-hexanoic acid, AMCHA). These agents have been extensively used in studies of fibrinolytic mechanisms and the conformational change in

the plasminogen molecule induced by EACA has been alluded to above. Trasylol (Aprotinin) is also an inhibitor of fibrinolysis which has been used in a similar fashion, but it is an extract of bovine lung and has a wide range of specificities. A full discussion of the mode of action of these inhibitors is beyond the scope of this thesis, but it has recently been suggested that these agents have their effect by reducing the binding of plasminogen and plasmin to fibrin[522].

MECHANISM OF THROMBOLYSIS IN VIVO

It is a widely held assumption that dissolution of whole blood clots and fibrin clots in vivo occurs via the plasminogen-plasmin system, although the contribution of other mechanisms has recently been explored (vide infra). The exact process of fibrin degradation within clots has attracted much interest and there are, at present, three hypotheses to explain physiological clot lysis.

Until recently, the theory of intrinsic clot lysis[4] was the most widely held view. According to this theory, lysis occurred as a result of the adsorption of plasminogen to fibrin in the clot, and the subsequent activation of inhibitor-free, gel phase plasminogen to plasmin in situ by activators which diffused through the clot. Evidence for this hypothesis was based on the known affinity of plasminogen for fibrin[563] which has recently been related to the lysine binding sites associated with the Kringle structures on the plasminogen molecule[479]. Plasmin formed while adsorbed to the fibrin molecule was free to digest the fibrin unimpeded by the presence of inhibitors, while any plasmin formed in the circulation was rapidly inactivated by inhibitors. A recent immunofluorescence study on thrombolysis in vivo has supported this hypothesis, by demonstrating

the specific adsorption of plasminogen to fibrin[216]. However, the same study also showed strong binding of plasminogen activator to fibrin, a finding which the authors did not discuss. In addition, previous workers had shown that the plasminogen content of thrombi was low[372] and that full lysis of artificial thrombi which were perfused with plasminogen activator did not occur unless plasminogen was present in the perfusate[328]. It is interesting to note that fast-acting antiplasmin interferes with the adsorption of plasminogen to fibrin[339].

The hypothesis of extrinsic clot lysis proposed by Ambrus and Markus[5] was based on the presumption that plasminogen was activated in the circulation and rapidly formed a complex with antiplasmin. Plasmin dissociated from the complex when it came into contact with fibrin because it had a greater affirmity for fibrin than for antiplasmin. However, evidence for this hypothesis is lacking, and in vitro clot lysis studies with purified plasmin have shown that such exogenous lysis occurs only very slowly. A modified view of this hypothesis has been given some support on the basis of fibrinogenolytic activity within the α_2 macroglobulin-plasmin complex[205].

A more recent hypothesis has been proposed which depends on the selective binding of plasminogen activator to fibrin[87]. In this situation, plasminogen diffuses through the clot and is converted to plasmin in the presence of high levels of activator.

It is probable that the answer to physiological clot lysis combines aspects of all three hypotheses. Thus some forms of plasminogen are strongly adsorbed to fibrin, while others are only weakly bound[505]. Similarly, the affinity of activators for fibrin is variable; for instance, tissue activator adsorbs to fibrin more readily than urokinase[506], and plasmin has a strong affinity for fibrin

and antiplasmin[168]. Furthermore, non-fibrinolytic mechanisms in fibrin degradation probably play a significant role in clot lysis, if not in the circulation, then at least in the tissues during wound repair. Considerable evidence now exists to show that leukocytes contain sufficient fibrinolytic and non-fibrinolytic proteases to digest fibrin (see above, plasminogen activators). Morphologically, fibrin deposits in the tissues induce a pronounced polymorphonuclear cell response[211,434] and in vitro plasma clot lysis with streptokinase is greatly enhanced by the addition of viable leukocytes[189]. It has been suggested that a prerequisite for leukocyte-induced thrombolysis is disintegration of the cells within the thrombus and release of their proteases[189]. It is also of interest to note that leukocyte fibrin-splitting neutral protease shows considerable differences from plasmin in its mode of digesting fibrin, and that it does not seem to be inhibited by synthetic inhibitors such as EACA[402], and only partially by anti-activators. However, natural inhibitors of leukocyte protease are present within the leukocyte itself and are also probably released on cell death[262]. A special role for the eosinophil leukocyte in the lysis of early fibrin deposits associated with platelets during the remodelling of thrombi, has also been suggested[276,435]. Current opinion on the role of leukocytes in fibrin dissolution favours the view that intact granulocytes[570] and macrophages[516] have no direct effect on fibrin, but do secrete plasminogen activator, whereas lysed cells can degrade fibrin directly[171].

FIBRINOGEN DEGRADATION BY PLASMIN

The structure of the fibrinogen molecule has been described in Chapter 2. When clotting occurs, fibrinogen is converted to fibrin with

the release of peptide material, designated Fibrinopeptides A and B. Plasmin is capable of degrading both fibrinogen and fibrin to soluble fragments generally known as fibrin(ogen) degradation products (FDP) (Figs. 5.4, 5.5). Digestion of fibrinogen yields Fragments D and E in the final stages. Intermediate Fragments X and Y are detectable after incomplete hydrolysis[166,171,312,347]. It can be seen that the process of fibrinogenolysis is asymmetric by the composition of Fragment Y[69]. In addition, one mole of fibrinogen will yield two moles of Fragment D and one of Fragment E, with one mole each of Fragment X and Y as intermediates (Fig. 5.4). The scheme shown in Fig. 5.4, however, is an over-simplification of the process. Apart from the known heterogeneity of fibrinogen itself (see Chapter 2), there is considerable variation in the exact structure of the FDP, particularly X and D. This may, in part, reflect different methods of purification, but is also due to variable degrees of proteolytic cleavage of fibrinogen by plasmins, particularly in the extent of lysis of the Aα chain, in the case of Fragment X[349] and the γ-chain remnant in the case of Fragment D[163]. Irregular plasmic digestion and interchange of disulphide bonds may also explain the heterogeneity of Fragment E[167]. A consequence of such varied digestion of fibrinogen is the diversity of the released peptide material, which may have significant biological activities in vivo (vide infra).

Digestion of fibrin by plasmin also yields FDP (Fig. 5.5), but of a different composition, depending on the degree of polymerisation in the molecule (see Chapter 2 for discussion of fibrin polymerisation). Non-crosslinked fibrin monomer is cleaved to produce Fragments X, Y, D and E, similar in structure to fibrinogen lysis products except that they no longer possess fibrinopeptides A and B[474]. Digestion of fully cross-linked fibrin yields the unique fragment D-dimer, and also Fragment E

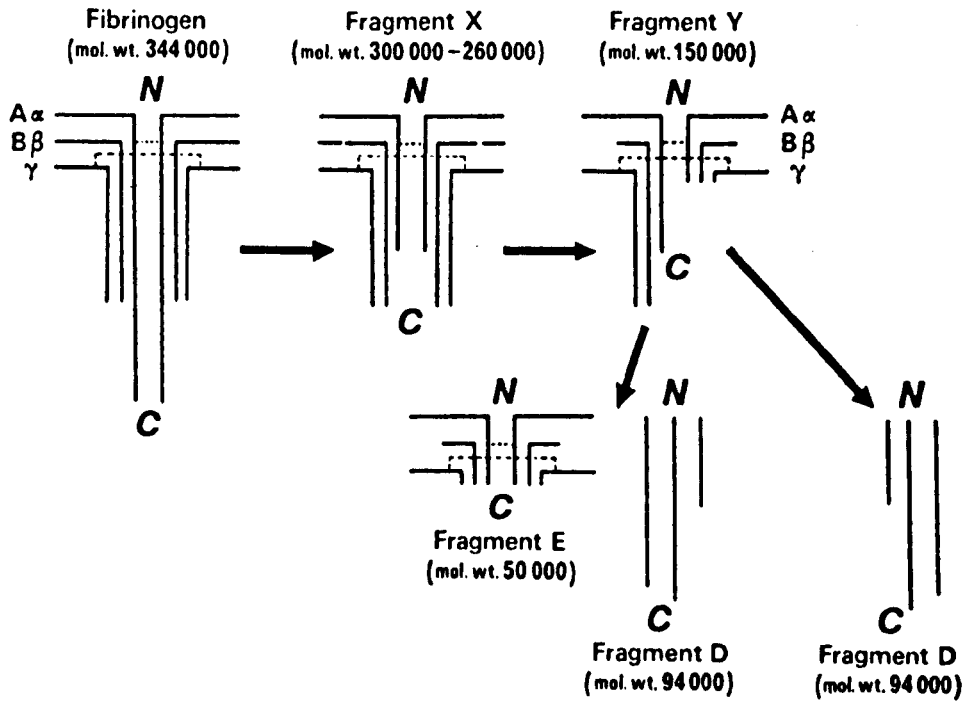


Figure 5.4 Schematic representation of the polypeptide chain origin of the constituent subunits of each fragment. α, β, γ refer to the individual chains of the fibrinogen molecule. A, B refer to the peptide fragment which is removed when fibrinogen is converted to fibrin. Dashed lines indicate interchain disulphide linkages. Figures refer to the molecular weights of the individual molecules. N,C represent the amino-terminal and carboxyterminal ends of the molecules respectively. (Gaffney[168]).

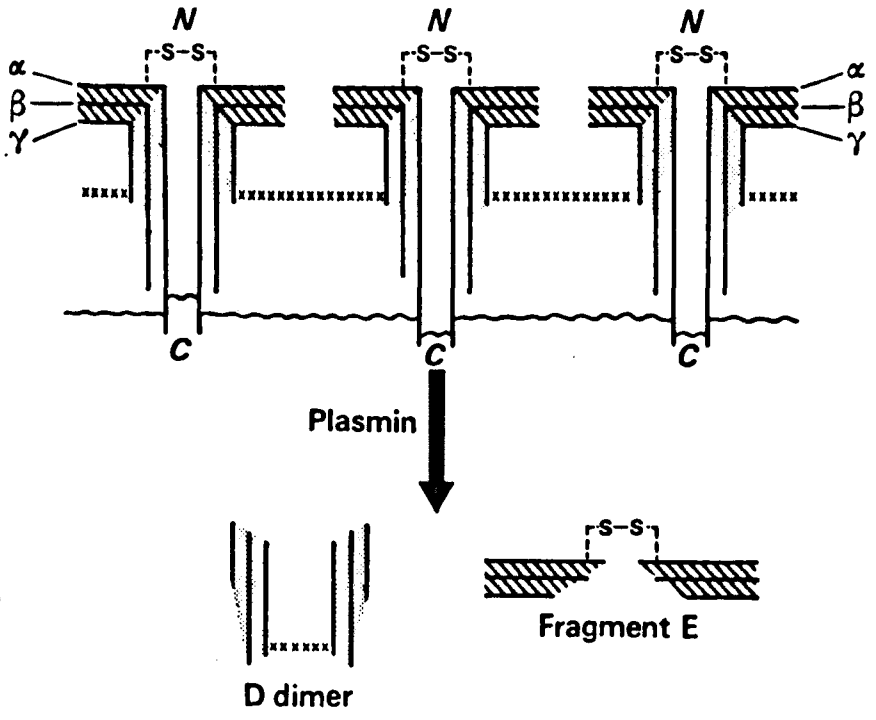


Figure 5.5 Fibrin polymerisation occurs via both the γ chain (xxxx) and the α-chain (∿∿) near the carboxy (C) terminal. Plasmin digestion of crosslinked fibrin yields D-dimer and E fragments as shown. Shaded areas represent intramolecular disulphide bonds between the α, β, and γ chains of each fibrin molecule (Gaffney[168]).

The D-dimer fragment contains crosslinked γ chains from different fibrin molecules, and it also may show heterogeneity[130]. Partially crosslinked fibrin, i.e. γ chain linkage without α chain polymerisation, also yields D-dimer and E fragments, but the lack of α polymers seems to make the molecule much more susceptible to lysis[170]. Recent studies by Rampling[414], however, have disputed the relationship between cross-linking of fibrin and lysability of clots, and suggest that the most important factor is uniform distribution of activator throughout the clot. Production of Fragments X and Y probably also occurs in fibrin digestion, but due to the extensive cross-linkages, they are probably not rendered soluble, but are retained within the structure of the clot. In addition, there is recent evidence to suggest that fragments larger than the fibrinogen molecule are released during the early stages of digestion of stabilised fibrin.

The view of fibrinolysis presented here is oversimplified, and indeed it has been shown that in addition to D-dimer and E, several other fragments may occur, such as D-dimer-E complex, D-monomer, and two as yet unnamed fragments[172].

ROLE OF FIBRINOLYSIS IN WOUND HEALING AND INFLAMMATION

It has long been recognised that fibrin formation is crucial for normal wound healing and recent studies have shown that a possible mode of action of fibrin in this respect could be by way of a common linkage protein, cold-insoluble globulin, which can cross-link to fibrin and also appears to reside on fibroblast surface membranes[343,447]. Another process which is central to normal wound healing is the production of an appropriate inflammatory response, and in this fibrinolysis may play a significant role. Generation of plasmin not only leads to removal of fibrin deposits, but due to the wide substrate specificity of plasmin

it promotes kinin formation[247] (and thus increased vascular permeability and vaso-dilation), it cleaves complement components C_3 and C_5[542] (thus increasing vascular permeability and promoting chemotaxis of leukocytes which secrete further plasminogen activators), and it produces FDP, which are themselves chemotactic[485]. Plasmin has other effects that may be relevant to wound healing, such as the ability to cleave the cold-insoluble globulin (fibronectin, LETS protein) discussed above[233]. Plasmin may also be mitogenic and thus contribute to cellular proliferation within wounds[544].

Finally, the biological activity of FDP may influence the inflammatory response. In addition to having chemotactic activity, FDP may inhibit lymphocyte transformation[271] and platelet aggregation[484]. They may enhance capillary permeability[68] and cause contraction of smooth muscle in vessel walls[307]. They may also have an immunosuppressive effect in neoplasia[176].

SUMMARY

From this brief review, it can be seen that fibrinolysis is a complex process which may have many effects apart from the dissolution of fibrin. It is clear that, although considerable advances have been made in the elucidation of the plasminogen-plasmin system and its interactions, much remains to be discovered, particularly in relation to plasminogen activators. Moreover, the exact mechanisms involved in thrombolysis are still largely unknown. To study the fibrinolytic process in isolation, however, is to ignore its roles in several other phases of pathophysiology such as wound healing and the inflammatory response. Recent studies have provided exciting new evidence of the complexities and far-reaching influence of this system, which may

have profound effects on several homeostatic mechanisms.

CHAPTER 6

MATERIALS AND METHODS FOR STUDY OF FIBRINOLYTIC MECHANISMS IN THE VITREOUS

INTRODUCTION

The techniques and reagents used for experiments relating to fibrinolytic mechanisms in the vitreous are described in this chapter. Two approaches were adopted in these studies. In the first, the fibrinolytic activity of the normal vitreous was investigated and for these experiments in vitro techniques were used. In the second phase of the study, fibrinolysis within the vitreous during the re-absorption of experimental vitreous blood clots was investigated. An in vivo rabbit model for vitreous clot lysis was established, and the extracted vitreous clots characterised for fibrin and FDP using biochemical and immunological techniques.

IN VITRO TECHNIQUES

MATERIALS

The anticoagulant used for collection of blood from rabbits by intracardiac puncture was acid citrate dextrose (0.085M sodium citrate; 0.065M citric acid; 0.2% dextrose). The proportions were one part ACD to eight parts whole blood. The blood was centrifuged at 2000G for 15 minutes and the supernatant plasma stored at -20°C. Human plasma was obtained from healthy volunteers, after collection of whole blood in 0.13M sodium citrate pH 7.0, and stored at -20°C.

Human plasminogen-rich fibrinogen was obtained from Kabi Pharmaceuticals, Stockholm, and was 90% protein clottable. Bovine plasminogen-rich fibrinogen was obtained from Sigma Chemical Co. Ltd., London, and contained 68% clottable protein.

Bovine thrombin 'topical' was obtained from Parke, Davis and Co., Detroit, Michigan, and was prepared as a stock solution of 100 NIH u/ml and stored at -20°C.

Streptokinase (Varidase; streptokinase/streptodornase 100,000 u/ml) was obtained from Lederle Laboratories, and stored at -20°C.

The following reagents were obtained from British Drug Houses, Poole, U.K.: casein, which was stored at -20°C as a 5 per cent solution in phosphate buffered saline; sodium azide; glycine; tetramethylethylene diamine puriss; acrylamide and bisacrylamide which were mixed as a stock solution of 22.5 per cent and 0.675 per cent respectively in distilled water and stored at 4°C; sodium dodecyl sulphate; β-mercaptoethanol; crystalline urea; Folin Ciocalteau's phenol reagent; and tyrosine.

Urokinase was supplied by Leo Laboratories Ltd., London.

Tris-saline buffer contained 0.05M tris (hydroxymethol) aminomethane-HCl and 0.01M NaCl, pH 7.4.

Veronal buffer (double strength) pH 7.2, was prepared by dissolving 4.1g sodium barbitone, 17.0g sodium chloride and 161.2 ml of N/10 hydrochloric acid in distilled water to a final volume of one litre.

Owren's buffer, pH 7.35, was prepared by dissolving 11.75g sodium diethyl barbitone, 14.7g sodium chloride, and 43 ml N hydrochloric acid in distilled water to a final dilution of two litres.

Phosphate-buffered saline, pH 7.6, was prepared as a solution of 0.25M disodium phosphate in 0.9% sodium chloride.

Polyacrylamide gel electrophoresis buffer, pH 7.2, was prepared by dissolving 8.82g of sodium hypophosphate, 25.3g disodium hypophosphate, and 2.0g of sodium dodecyl sulphate in distilled water to a final dilution of one litre.

Agar was obtained from Difco Laboratories, Detroit, Michigan.

Trasylol was obtained from Bayer Ltd., Germany.

Ammonium persulphate was used as a 1.5% solution in distilled water.

Anti-rabbit fibrinogen serum was obtained from Hoechst Pharmaceuticals Ltd., Germany.

Sepharose-4B was obtained from Pharmacia, Uppsala, Sweden.

METHODS

All centrifugations were done in an International PR-2, Servall RC-2 or MSL-6L centrifuge at $0^{o}C$ to $4^{o}C$. Ultracentrifugation of vitreous samples was performed in a Beckman L5-50 ultracentrifuge at $0^{o}C$ to $4^{o}C$.

Vitreous samples

Samples of vitreous were obtained as described in Chapter 3. Studies of the fibrinolytic activity of normal vitreous from human, dog and sheep eyes were performed on samples obtained by the aspiration and dissection techniques (p. 39) while vitreous samples from rabbit eyes containing blood clots (see later) were obtained by the freezing method (p. 40).

Estimation of fibrinolytic activity

Two methods were used in these experiments; the fibrin plate method of Nillson and Olow[365] and the euglobulin lysis time[67].

(a) The Fibrin Plate method

This method provides a semiquantitative assessment of plasminogen activator activity in the presence of constant levels of plasminogen

and fibrinogen. The method is based on measuring the areas of lysis produced in standard plasminogen-rich fibrin layers by fibrinolytically active materials, which are placed as drops on the surface of the fibrin. The reproducibility and accuracy of the method have been demonstrated[365].

1 ml aliquots of a plasminogen-rich fibrinogen solution (250 mg/ml) in distilled water were diluted with 24 mls of Tris buffer and poured into planar perspex dishes (12 cm diameter). The solution was clotted with 0.5 ml bovine thrombin (50 NIH u/ml) using constant agitation to ensure thorough mixing of the solutions. The plates were allowed to become firm by incubating at 4°C for one hour.

Test samples (0.03 ml) were pipetted onto the surface of the fibrin plates and incubated at 37°C for 24 hours. Areas of lysis were measured and fibrinolytic activity of the samples related to a standard curve, using urokinase as the standard activator (see Chapter 7).

(b) Euglobulin lysis time

This method is based on the assumption that the euglobulin precipitate of plasma is free of inhibitors of fibrinolysis[67]. Consequently, the lysis time of a clot prepared from the redissolved euglobulin precipitate containing plasminogen and fibrinogen, is a reflection of plasminogen activator activity within the plasma sample. The method is used to test fresh plasma samples for plasminogen activator activity, since plasma activator is considerably labile. By using stored plasma, which has minimal activator activity, the technique can be modified to test for activator activity in samples added to the euglobulin precipitate before clotting.

Euglobulin precipitates were prepared from human plasma by adding 9.5 mls of 0.014% acetic acid to 0.5 ml human plasma. The tubes were

inverted and allowed to stand for 10 minutes at 4°C. After centrifugation at 6000G for ten minutes, the precipitates were redissolved in 0.5 ml Owren's buffer, and vitreous or buffer added. The samples were clotted with 0.5 ml thrombin (2 NIH u/ml) and incubated at 37°C. The time necessary for the clot to dissolve was noted.

Fibrinogen estimation

Fibrinogen was estimated in samples of vitreous by the method of Ratnoff and Menzie[417]. In principle, this method requires prior clotting of fibrinogen within the sample by an excess of thrombin; the fibrin concentration is then measured by a protein assay using Folin Ciocaltean's phenol reagent and a tyrosine standard (200 mg/l 0.1N HCl). 0.2 ml vitreous was added to 10 mls 0.9% saline in crushed glass and 0.05 ml bovine thrombin added. The reaction proceeded for 10 minutes at 4°C after which the samples were centrifuged at 2000 r.p.m. for 5 minutes. The supernatant was removed and the glass beads washed twice with saline. One ml of 10% NaOH was added to the beads and the samples boiled for ten minutes. Seven mls distilled water and 3 mls 20% Na_2CO_3 were added and the samples thoroughly mixed. The reaction was completed with the addition of 1 ml Folin's reagent. After 30 minutes at room temperature, the samples were centrifuged at 2000 r.p.m. and the absorbance at wavelength 650 nm read on a SP6-400UV spectrophotometer (Pye Unicam) and compared with a standard tyrosine solution. The concentration of fibrinogen (f) was calculated from the formula:

$$f\ (mg/100\ ml) = \frac{A^{650}\ sample}{A^{650}\ standard} \times 11.7 \times 500 \times \frac{1}{10}$$

Plasminogen estimation

Plasminogen was assayed by the method of Remmert and Cohn[426] as

modified by Alkjaersig et al[4]. In principle, this method is based on the conversion of plasminogen to plasmin, which is then measured by caseinolysis. Acid-treated plasma and vitreous samples (0.5 ml N/6 plus 0.5 ml sample for 15 minutes; reaction stopped with 0.5 ml N/6 NaOH, followed by 1 ml 0.25M phosphate buffer) were added to 0.5 ml streptokinase and the mixture incubated with 2 ml casein at 37°C (total volume 5 mls). After two minutes, 2 mls of the sample was withdrawn and added to 2 ml of 10% trichloracetic acid. After 62 minutes, a further 2 ml of the sample was withdrawn and treated likewise. Both tubes were centrifuged at 600G for 10 minutes, and one ml of the supernatant was added to 1.5 ml of diluted Folin's reagent (1 part: 3 parts of distilled water). The reaction was allowed to proceed for 30 minutes, and the extinction at 650 nm read. The values were compared to known values for standard tyrosine solution as for fibrinogen assay.

Estimation of α2-macroglobulin, α1 antitrypsin and fibrin degradation products

The radial immunodiffusion technique of Mancini, Carbonera and Hergmans[308] was used for the quantitative estimation of these proteins in vitreous samples. The method is based on the diffusion of proteins in agar gels which have been impregnated with specific antiserum. Protein solutions in wells cut in the agar gels diffuse outwards in a radial fashion and produce immunoprecipitation rings which are measured visually. The concentration of protein in the solution is directly proportional to the diameter of the ring.

Six mls of 0.9% saline containing antiserum to the protein was mixed with 6 mls of 2% agar in double strength Veronal buffer at 56° for a few minutes, before being poured into a plastic petri dish, diameter

8.5 cms, depth 1.5 cm. 5 µl samples were placed in wells in the agar gel, together with dilutions of standard protein solution whose content of the appropriate protein were known. The plates were incubated in a moist chamber for 48 hours at 40°C and the diameter of the precipition rings measured after staining with 10% tannic acid. A standard curve for the square of the diameter of the precipition ring against the concentration of the protein was drawn, and the values for the unknown vitreous samples interpolated from the curve. Minor modifications in the technique were required for different experiments and these are described in the appropriate sections.

Fibrin analysis

The detection and quantitation of fibrin within vitreous samples was carried out by polyacrylamide gel electrophoresis in sodium dodecyl sulphate, as described by Weber and Osborne[545]. This method is generally regarded as an accurate and quantitative method for identifying proteins in solution on the basis of their molecular weight. The gels were prepared at 7.5% acrylamide concentration by mixing together 0.045 mls tetramethylethylene diamine puriss, 1.5 mls 1.5% ammonium persulphate, 3.5 mls distilled water, 10 mls of a stock solution of 22.5% acrylamide/ 0.675% bisacrylamide and 15 mls gel buffer in the order stated. The solution was applied to glass cylinders (0.5 cm x 15 cm) and allowed to stand for 45 minutes at room temperature. The cylinders were fitted to a disc electrophoresis column rack (Shandon Ltd., London) and 2 µl Bromophenol blue applied to the top of each cylinder as a reference dye. 20 µl samples of vitreous extracts and standard solutions of known molecular weight were then applied to the top of each gel, and electrophoresis proceeded at 7-8 milliamps per tube for four hours, in the presence of gel buffer diluted one to one with distilled water. Power was supplied with

a Savant Power Pack (Hocksville, New York). After electrophoresis, the gels were stained with 0.45% coomassie brilliant blue in 7% glacial acetic acid for 2-4 hours and destained in 7% acetic acid for 24-48 hours. Fibrinogen and fibrin were identified within vitreous samples by comparing gel bands with standard solutions, and quantitated by densitometric scanning of the gels at 600 nm on a Gilford spectrophotometer with scanning attachment. The γ dimer peak heights were compared with known concentrations of crosslinked rabbit fibrin.

Preparation of rabbit fibrinogen

10 μl of Trasylol (10,000 u/ml) was added to 10 mls citrated rabbit whole blood, and the plasma withdrawn after centrifugation at 2000G for 10 minutes. An equal volume of a 25% saturated solution of ammonium sulphate was added, and the precipitate recovered after further centrifugation. The sample was redissolved in saline, and the procedure repeated. Precipitation was then effected with an equal volume of 2.7M glycine, and this material dissolved in Tris buffer, after recovery of the precipitate by centrifugation.

Preparation of rabbit fibrin

Rabbit fibrin clots were prepared from fresh rabbit plasma by incubating citrated plasma with bovine thrombin (10 NIH u/ml) in the presence of calcium ions at 37^{o}C, and allowing the reaction to continue for 2 hours to ensure crosslinking of the fibrin. The clot was thoroughly washed for 24 hours in 0.9% saline, and prepared for gel electrophoresis in the same manner as the vitreous extracts (see below).

Preparation of Rabbit Haemoglobin

Rabbit red cells were obtained from whole blood after centrifugation of citrated blood at 400G for 5 minutes. The leukocyte-rich

plasma was aspirated and discarded. The red cells were lysed with distilled water and the sample centrifuged at 3000G for 20 minutes. The haemoglobin-rich supernatant was then applied to a column of sepharose-4B (30 cm high by 1.7 cm diameter), previously equilibrated with Tris-buffer, and calibrated with protein solutions of known molecular weight. The sample was eluted with Tris buffer and the eluate corresponding to the haemoglobin peak (mol. wt. 50,000 to 100,000) collected and run on SDS-polyacrylamide gel electrophoresis as described above.

IN VIVO TECHNIQUES

MATERIALS

Animals

White New Zealand rabbits weighing 3-4 kgs were used.

Anaesthetics

Pentobarbital sodium (Abbott Ltd., London) was used for intravenous administration while topical anaesthesia was achieved with Amethocaine 1% eye drops (Smith and Nephew Ltd.).

Other ocular solutions

Chloramphenicol 0.5% and Atropine 1% eye drops were obtained from Smith and Nephew Ltd.

METHODS

Anaesthesia

The rabbits were anaesthetised by the intravenous infusion of sodium pentobarbital (20 mg/kg weight) through the marginal ear vein. Anaesthesia was considered adequate when the corneal reflex was no longer elicited, and was maintained for the duration of the experiment by intermittent infusion of pentobarbital and the application of topical amethocaine drops. Recovery from anaesthesia usually occurred 30–60 minutes after the experiment was complete.

Induction of vitreous clot

The eyelids were retracted with a speculum and the right eye of each animal was softened by withdrawing 0.15–0.20 ml aqueous from the anterior chamber using a 25-gauge needle inserted through the limbus. The eye was immobilised by fixing the superior rectus muscle and 0.2 ml whole blood was withdrawn from the marginal ear vein into a silicone-coated plastic syringe. Under ophthalmoscopic control, the blood was injected immediately into the centre of the vitreous gel through the pars plana in the anterosuperior quadrant of the globe with a 27-gauge needle. A short pause was taken before the needle was sharply withdrawn from the eye. No tracking of blood under the conjunctiva occurred, and it was assumed that the entire volume of blood was retained within the vitreous. Chloramphenicol 0.5% and Atropine 1% drops were instilled into the lower fornix at the end of the operation. 2–3 hours following the operation, the anterior chamber was usually reformed, although in some cases a longer time elapsed before it was re-established to its normal depth.

Ophthalmoscopic Observations

The resolution rate of the vitreous clot in vivo was followed by ophthalmoscopy at weekly intervals. The degree of opacification of the vitreous was arbitrarily scored according to the scheme shown in Table 6.1.

Preparation of the vitreous extract for FDP estimation and gel electrophoresis

At various stages throughout the course of the experiments, rabbits were sacrificed and the eyes enucleated immediately and transported to the laboratory on ice. The entire vitreous was removed by the freezing technique (p. 40) and centrifuged at 2000G for five minutes. 10 µl of Trasylol (10,000 u/ml) and 10 NIH units of bovine thrombin were added to the supernates which were recentrifuged at 1750G for 15 minutes and stored at -20°C. The residual solid vitreous clot was washed for 24 hours in 0.85% saline, and then dissolved in 1 ml of 8M urea. To each sample, 0.1 ml of a ten per cent solution of sodium dodecyl sulphate plus a similar volume of ten per cent β-mercaptoethanol were added. The samples were incubated at 37°C for 24-48 hours, and run on polyacrylamide gels as described.

Histology

Selected eyes from various animals were fixed in 2-4% phosphate-buffered glutaraldehyde for 24-48 hours and processed for histology. The method is fully described in Chapter 11.

TABLE 6.1

CLINICAL STAGING OF VITREOUS OPACITY

Stage 1	Completely opaque vitreous
Stage 2	Slight increase in red reflex, but no fundal detail visible
Stage 3	Partly visible fundal details, through gaps in vitreous opacity
Stage 4	Central vitreous clear; residual small opacities present
Stage 5	Completely clear vitreous.

CHAPTER 7

FIBRINOLYTIC ACTIVITY OF THE NORMAL VITREOUS

INTRODUCTION

Fibrinolytic activity within ocular tissues has been described by several authors and, as for other organs, the degree of activity of any one ocular tissue can be correlated with its vascularity[273]. Thus, the choroid and the retina are known to contain high levels of activity while the lens and the vitreous have been described as fibrinolytically inactive[273,382]. It should be noted that this relationship does not hold true for all ocular tissues, since the avascular cornea contains plasminogen activator activity, located within the epithelium[382], a fact recently confirmed by tissue culture experiments[279]. Release of this activity is apparently related to cell desquamation[383]. In addition, a fibrinolytic activator is present in primary and plasmoid aqueous humour and in the canal of Schlemm[452].

A relationship between the level of fibrinolytic activity and the rate of tissue repair within the eye has been proposed. Thus, the choroid and retina, which are rich in plasminogen activator[278,382] undergo rapid healing when injured. Aqueous fibrinolytic activity may also have a role in keeping the outflow pathways free of obstruction[383] and in clearing blood and inflammatory exudates from the anterior chamber[452]. By contrast, the slow resolution of vitreous blood clots has been attributed to low fibrinolytic activity within the sclera[273], but is more likely to be due to lack of activity within the vitreous itself. Although the existence of a "vitreous proactivator" of fibrinolysis has been postulated[423], earlier studies suggested that the vitreous does not contain activator activity[273,382]. The present study was therefore undertaken to determine whether components of the fibrinolytic system were present in normal vitreous. The study included assays

for plasminogen, fibrinogen, plasminogen activator and inhibitors of fibrinolysis.

MATERIALS AND METHODS

VITREOUS SAMPLES

Seven human, ten dog and seven sheep eyes were used for these experiments. Vitreous samples were obtained by the aspiration technique (Chapter 3). To exclude all possibility of contamination from other ocular structures, fluid vitreous samples were centrifuged at 4000 r.p.m. for 20 minutes at $4^{o}C$, and only the crystal clear supernatant vitreous humour used for estimation of fibrinolytic activity.

PLASMINOGEN and FIBRINOGEN

These were assayed by the methods described in Chapter 6.

PLASMINOGEN ACTIVATOR

Plasminogen activator activity was assessed in samples of vitreous by the fibrin plate technique, and by a modified euglobulin lysis time using stored human plasma. The principles on which these methods are based are discussed in Chapter 6.

Fibrin plate method

Bovine and human fibrin plates were prepared as described (Chapter 6). Triplicate samples of vitreous (0.03 ml) were pipetted

onto the fibrin layer and the plates incubated for 24 hours at 37°C. Heated fibrin plates and plates containing e-amino-caproic acid (EACA) a synthetic inhibitor of fibrinolysis, at 10^{-2} molar concentration were also used. Tests were also performed on samples of vitreous which had been incubated for varying lengths of time at room temperature and at 37°C. Plasminogen concentrations in the fibrinogen preparations were assayed as described (Chapter 6). For standardisation of fibrinolytic activity, known concentrations of a reference standard solution of urokinase were tested in triplicate on human and bovine fibrin plates and equivalent mean values for lysis areas of vitreous interpolated on a log/log graph.

Modified Euglobulin Lysis Time

The euglobulin lysis time is a measure of the concentration of inhibitor-free plasminogen activator in fresh plasma. Using stored plasma, which has little or no plasminogen activator activity, euglobulin precipitates were prepared as described (Chapter 6) and redissolved in 0.5 ml 0.01M Owren's buffer. 0.5 ml vitreous was added and the mixture clotted with 0.5 ml thrombin, 1 NIH unit/ml. Clot lysis times were then compared with controls using 0.5 ml 0.01M Owren's buffer in place of vitreous. A further control was used which combined EACA at 10^{-2}M concentration with the euglobulin precipitate.

INHIBITORS OF FIBRINOLYSIS

The concentration of inhibitors of fibrinolysis within human vitreous samples was estimated by the radial immunodiffusion technique[308] (Chapter 6). Commercial diffusion plates were used (Partigen, London) in which antisera to α_1 antitrypsin and α_2 macroglobulin were incorporated into the 1% agar support at a 4% concentration. Duplicate 5 µl samples

of human vitreous were placed in wells in the agar, and the inhibitor concentrations estimated by measuring the diameter of the immuno-precipitation rings after 48 hours and comparing this value with a standard curve of known protein concentration (Chapter 6).

RESULTS

PLASMINOGEN and FIBRINOGEN

Neither of these proteins was detected in vitreous samples from any of the species tested, using the present assay methods.

PLASMINOGEN ACTIVATOR

Fibrin plate method

Areas of lysis were observed in human and bovine fibrin plates for human, dog and sheep vitreous (Fig. 7.1) and the results expressed in equivalent Ploug units of urokinase as described in Materials and Methods (Tables 7.1, 7.2, Fig. 7.2). Some species difference in activator activity occurred since slightly higher levels were noted in human than in dog or sheep samples. Activator levels in the vitreous, however, were always low, ranging from 10-30 Ploug units of urokinase per ml of vitreous. Areas of lysis on bovine fibrin plates were greater than on human fibrin plates, but this may have been a reflection of the higher concentration of clottable protein in human fibrin (Chapter 6). The plasminogen concentration of bovine and human fibrinogen used in this study were 0.70 and 1.13 casein units respectively.

No lysis was obtained on heated plates, nor on plates containing EACA. This indicated that the activity was due specifically to

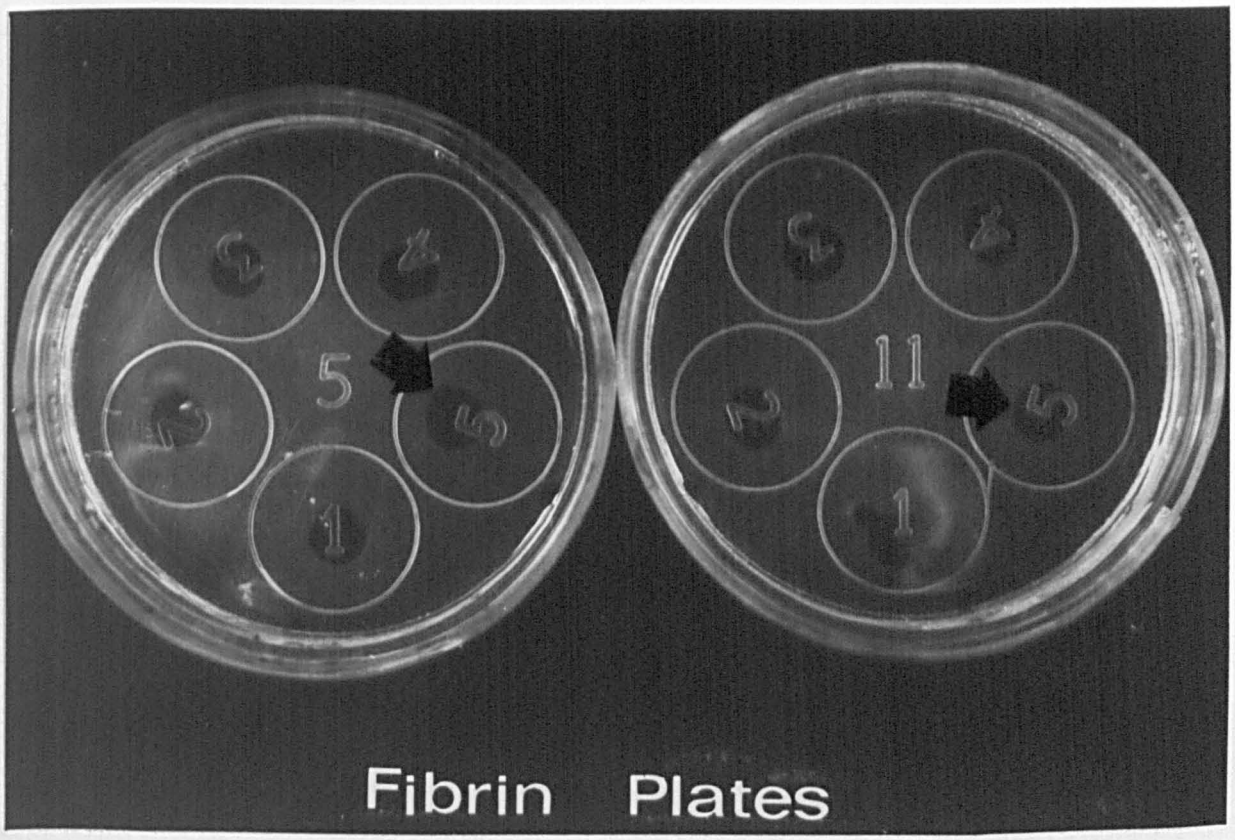


Figure 7.1 Bovine fibrin plates were incubated at 37°C for 24 hours with human vitreous samples. Areas of lysis on the fibrin plates are denoted by the clear zones (arrows).

RESULTS

TABLE 7.1

Vitreous plasminogen activator activity on human fibrin plates

No. of Eyes	Human	Dog	Sheep
1	0.61*	0.46	0.38
2	0.60	0.66	0.34
3	0.56	0.46	0.47
4	0.63	0.55	0.37
5	0.61	0.58	0.38
6	0.60	0.63	0.52
7	0.55	0.64	0.52
8	-	0.45	-
9	-	0.59	-
10	-	0.49	-
Mean	0.59	0.55	0.43
Standard dev.	0.028	0.08	0.08
S.E.M.	0.01	0.03	0.03

Activator activity is expressed as equivalent Ploug units of urokinase interpolated from Fig. 7.2.

*Values represent mean of triplicate samples.

P = 1.35 (human vs dog)

P = $<$ 0.0005 (human vs sheep)

TABLE 7.2

Vitreous plasminogen activator activity,

on bovine fibrin plates

No. of eyes	Human	Dog	Sheep
1	0.61	0.31	0.91
2	0.65	0.39	0.61
3	0.49	0.37	0.44
4	0.62	0.35	0.41
5	0.68	0.36	0.56
6	0.51	0.42	0.34
7	0.58	0.47	0.36
Mean	0.59	0.38	0.51
Standard dev.	0.07	0.05	0.19
S.E.M.	0.03	0.02	0.07

Activator activity is expressed as equivalent Ploug units of urokinase interpolated from Fig.2.

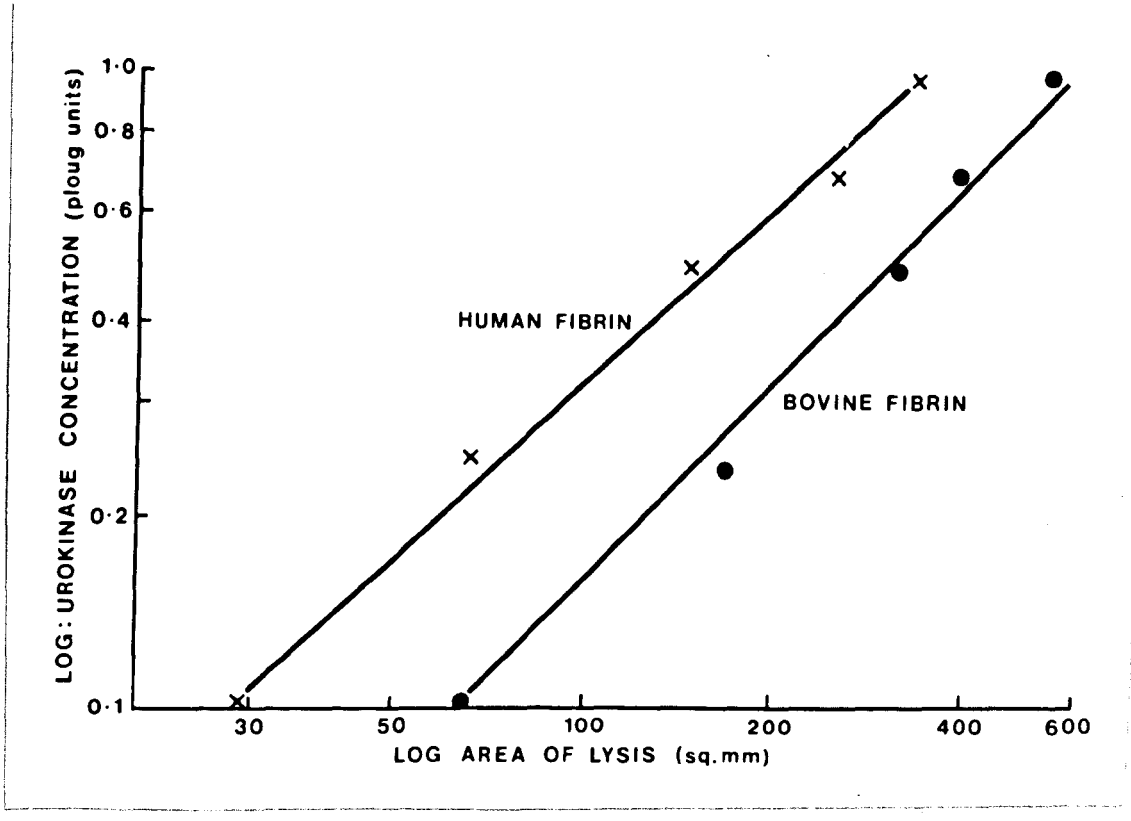


Figure 7.2 Standard log curves for areas of lysis on human and bovine fibrin plates with a reference urokinase solution scale.

plasminogen activator within the vitreous, and excluded the presence of other protease or plasmin activity.

A time-course study indicated that the degree of lysis was proportional to the time of incubation of vitreous with fibrin on a logarithmic basis (Fig. 7.3). However, no lysis was seen for the first two hours of incubation, which possibly indicated complex enzymatic relationships during this period.

The rate of disappearance of plasminogen activator activity within the vitreous of dogs was assessed following incubation of the vitreous for varying lengths of time at room temperature and at 37°C (Tables 7.3, 7.4). Some reduction in activator activity was observed at room temperature, which indicated that vitreous plasminogen activator was predominantly stable, but contained a labile component.

Modified Euglobulin Lysis Time

The clot lysis times noted when vitreous was incubated with stored plasma euglobulin precipitates were considerably shorter than in controls using buffer (Table 7.5). Lysis times in the controls represent the diminished activator activity of the stored plasma; therefore, the shortened lysis times of the vitreous samples suggest the presence of plasminogen activator within the vitreous. When EACA was combined with the euglobulin-vitreous mixture, no lysis occurred after 24 hours. This further supported the evidence that the fibrinolytic moiety within the vitreous is indeed plasminogen activator.

INHIBITORS OF FIBRINOLYSIS

Samples of human vitreous (5 µl) failed to produce a precipitin ring against antisera to human α_2 macroglobulin in radial immunodiffusion

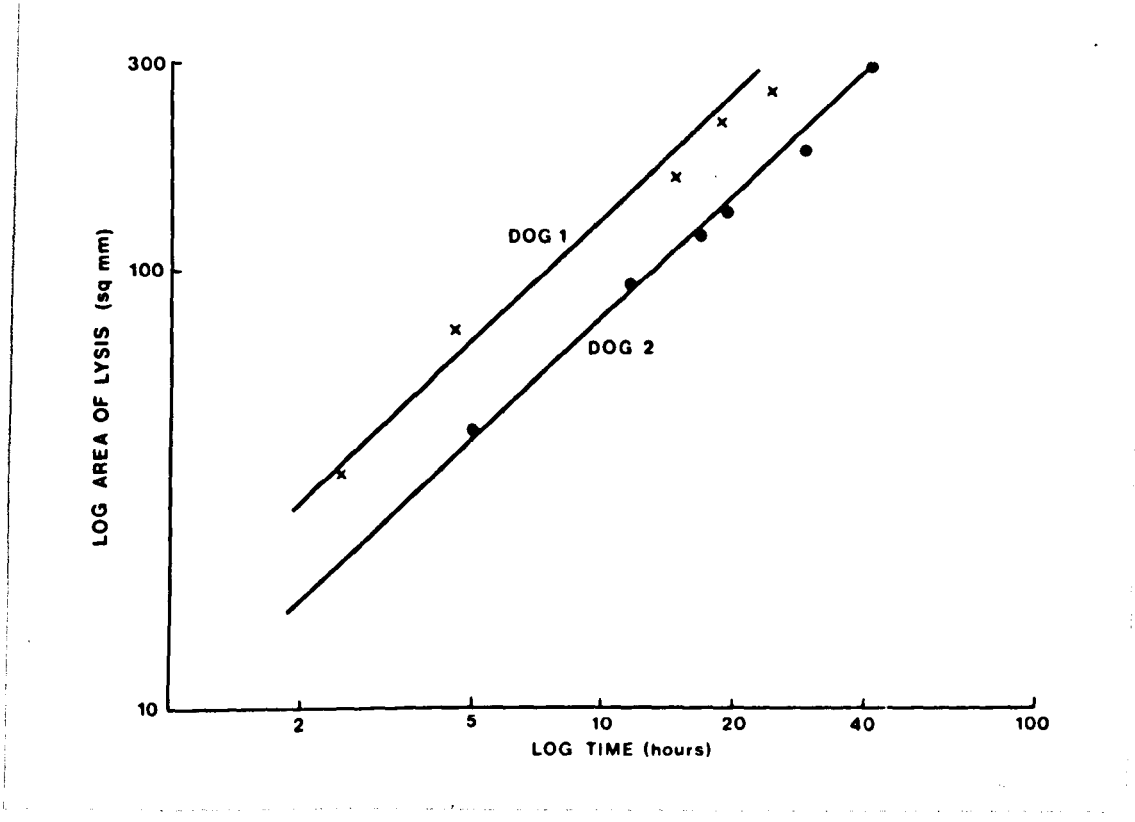


Figure 7.3 Areas of lysis due to vitreous samples on human fibrin plates were first detectable after approximately two hours and increased linearly with time when plotted on a log/log graph.

TABLE 7.3

Vitreous plasminogen activator activity observed following incubation of samples at room temperature

No. of Eyes (Dog)	Incubation period (hours) 0	3	6	24	48
1	0.46	0.0	0.33	0.36	0.12
2	0.53	0.31	0.36	0.35	0.24
3	0.61	0.54	0.17	0.24	0.35
4	0.61	0.47	0.48	0.42	0.37
5	0.48	0.53	0.50	0.44	0.41
6	0.46	0.48	0.45	0.62	0.48
7	0.53	0.51	0.50	0.57	0.35
8	0.46	0.44	0.51	0.49	0.39
Mean	0.52	0.41	0.41	0.43	0.34*
Standard dev.	0.06	0.18	0.11	0.12	0.11
S.E.M.	0.02	0.06	0.04	0.04	0.04

* $P < 0.001$ (time 0 vs time 48)

Activator activity is expressed as equivalent Ploug units of urokinase interpolated from Fig.2.

TABLE 7.4

Vitreous plasminogen activator activity observed following incubation of samples at 37°C

No. of Eyes (dog)	Incubation period (hours) 0	3	6	24	48
1	0.60	0.44	0.53	0.69	0.57
2	0.72	0.40	0.60	0.59	0.60
3	0.64	0.47	0.38	0.44	0.59
4	0.61	0.42	0.49	0.44	0.57
5	0.51	0.53	0.50	0.53	0.59
6	0.53	0.48	0.56	0.42	0.79
7	0.53	0.50	0.53	0.47	0.49
Mean	0.59	0.46	0.51	0.51	0.60
Standard dev.	0.07	0.05	0.07	0.09	0.09
S.E.M.	0.03	0.02	0.03	0.40	0.03

Activator activity is expressed as equivalent Ploug units of urokinase interpolated from Fig.2.

TABLE 7.5

Modified euglobulin lysis time

No.of Eyes (Dog)	Clot lysis time (hours) Vitreous sample	Buffer Control
1	1.30	15.34
2	1.75	16.25
3	0.75	6.45
4	1.25	7.25
5	1.05	7.40
6	1.25	8.25
7	1.30	9.25
Mean	1.23	10.03
Standard dev.	0.30	4.04
S.E.M.	0.11	1.53

Significance $P < 0.0005$

plates, although a faint immunoprecipitation line was detected between human vitreous and α_1 antitrypsin. Quantitation against the standard concentration curve indicated that the total α_1 antitrypsin-like protein concentration within the vitreous was 10 ± 2.3 µg/ml.

DISCUSSION

The presence of activator activity within the vitreous, as shown by this study, contrasts with the findings of Pandolfi, Coccheri and Astrup[382] and Kwaan and Astrup[273]. Using a slightly different fibrin plate technique[382] and Todd's histochemical technique[273], they reported high activity in the choroid, moderate activity within the retina and no activity within the vitreous body. Their samples were incubated for a shorter period of time at 37°C than the samples in this study. However, this study has shown that lysis of fibrin under the influence of vitreous activator usually begins within one to two hours of incubation at 37°C (Fig. 7.3) and by twelve hours, appreciable lysis has occurred. It is probable that the present observations of vitreous activator reflect the improved sensitivity of the fibrin plate method of Nillson and Olow[365].

Activation of the plasminogen-plasmin system in vivo may be achieved by blood or tissue activator, each of which is identifiable by its physical characteristics (see Chapter 5). Blood activator is labile and is rapidly destroyed at room temperature, whereas tissue activator, while having a labile component, is for the greater part thermostable, even at acid pH[15]. Tables 7.3 and 7.4 show the relative stability of vitreous activator at room temperature and at 37°C. It is possible that the slight reduction in activity which was observed with vitreous activator at room temperature may represent the labile component of tissue activator, or it may indicate that vitreous activator is partly derived from blood activator. However,

a greater proportion of the activity was retained at room temperature. In addition, vitreous activator caused lysis of human and bovine fibrin, and as such conformed with the activity of direct tissue activator, unlike indirect activators which only cause lysis of human fibrin. In this respect, vitreous activator differs from that found in aqueous, which causes lysis of human fibrin only[452]. Thus, it appears that vitreous activator is true tissue activator. In view of the nature of the vitreous body, i.e. a modified connective tissue, it would seem reasonable that its content of plasminogen activator should derive from tissue activator.

Current theory suggests that the source of tissue activator is the endothelium of blood vessels, particularly those in actively growing tissues (see Chapter 5). Since the vitreous is avascular, plasminogen activator within this tissue may be produced by the surrounding ocular tissues and thence enter the vitreous by diffusion. However, tissue activator is strongly cell-bound and is not readily released by endothelial cells, except when cell death occurs[13]. In addition, it would be expected that diffusion of activator into the vitreous would be retarded by the molecular sieve effect of the vitreous macromolecules, such as hyaluronic acid[403]. Since the molecular weight of tissue activator occurs within the range of 57,000-80,000 (see Chapter 5), this retardation effect would be quite marked.

An alternative hypothesis is that the plasminogen activator of vitreous is related to its fixed tissue cells, since many cell types including blood and epithelial cells, are known to synthesise and secrete plasminogen activator (see Chapter 5). Freeman et al[157] have demonstrated proteolytic activity related to the lysosomal and microsomal fractions of vitreous cells, and it has been further shown that such organelles are associated with the production of plasminogen activator[277]. In fact,

the paucity of cellular elements in the vitreous may be directly related to the low levels of fibrinolytic activity in the vitreous.

The low concentration of fibrinolytic components within the vitreous may have considerable bearing on the physiological removal of blood and fibrin deposits. Clearly, the virtual absence of inhibitors of fibrinolysis would promote a more rapid removal of such material. It is also worth noting that the absence of inhibitors may be further compounded by the "peptone" effect, whereby inhibitors of fibrinolysis introduced into the vitreous during bleeding may be effectively removed by the polyelectrolyte, hyaluronic acid, as proposed by Astrup and Rosa[14] (See Chapter 5, "peptone effect" of acid polysaccharides). However, the very low levels of plasminogen activator activity (10 to 30 equivalent Ploug units of urokinase per ml in the present study, see Tables 7.1, 7.2) would militate against a rapid clearance of blood clots.

SUMMARY

The vitreous bodies of human, dog and sheep eyes were examined for the presence of components of the fibrinolytic system. Fibrinogen, plasminogen, and α_2 macroglobulin were not detected in normal vitreous, and only trace levels of α_1 antitrypsin were found. Plasminogen activator activity of normal vitreous was also found to be low (10-30 equivalent Ploug units of urokinase per ml of vitreous) and it is suggested that this may contribute to the delayed resolution of vitreous haemorrhage in vivo.

CHAPTER 8

IN VIVO FIBRINOLYSIS WITHIN EXPERIMENTAL VITREOUS BLOOD CLOTS

INTRODUCTION

It has been shown that blood will clot in the presence of vitreous in vitro (Chapter 4) and other studies suggest that, at least in the experimental situation, clotting will also occur in vivo when the blood invades the vitreous gel[223,421,458,490]. The evidence for this is based mainly on the histological demonstration of fibrin within vitreous blood clots[223, 458]. While considerable attention has been paid to such processes as haemolysis[196], phagocytosis[224] and membrane formation (see Chapter 13) within resolving vitreous clots, fibrinolytic mechanisms have rarely been studied. Regnault[422] injected ^{125}I-labelled human fibrinogen into rabbit vitreous and found that the half-life of fibrinogen was four days, with no trace of radioactivity after twenty days. He suggested that "spontaneous lysis" of fibrin was the initial step in the resolution of vitreous clots.

In the previous chapter, it was shown that, although plasminogen activator activity was present in normal vitreous, the level of activity was low, and it was suggested that this was a factor in the delayed resolution of vitreous clots. The object of the experiments described in this chapter was to provide biochemical evidence for the presence of fibrin within vitreous blood clots in vivo, and to study the process of fibrinolysis by recording the progressive removal of fibrin from the vitreous and by searching for intravitreal soluble FDP.

METHODS

ANIMAL MODELS

White New Zealand rabbits (300-400 kg) were used. The method of

anaesthesia and the induction of vitreous blood clots has been described in Chapter 6. The resolution of the vitreous clots was monitored weekly by ophthalmoscopy for a period of eight months following the injection of blood into the vitreous. Rabbits were sacrificed in pairs at various time intervals, and the globes enucleated immediately and transported on ice to the laboratory.

PREPARATION OF THE VITREOUS EXTRACT

This has been described in Chapter 6. The normal vitreous of the fellow eye was removed in similar fashion and served as a control.

FIBRIN ANALYSIS

The detection and quantitation of fibrin within the vitreous clot was carried out by SDS-polyacrylamide gel electrophoresis, using 7.5% acrylamide gel as described in Chapter 6.

PREPARATION OF STANDARDS

Control samples of rabbit fibrinogen, fibrin and haemoglobin were prepared as described in Chapter 6. These samples were run on gels as standards, as were samples of normal rabbit vitreous and streptokinase-plasmin-incubated vitreous clots.

DETECTION OF FDP

The concentration of FDP within the supernatant from the vitreous clot was estimated by the radial immunodiffusion technique (see Chapter 6). Anti-rabbit fibrinogen serum was incorporated into the 1% agar

support at a concentration of 0.4%.

PLASMINOGEN ACTIVATOR ACTIVITY

The fibrin plate technique was used to measure plasminogen activator activity of the supernatant from vitreous clots, while the normal vitreous from the fellow eye served as the control.

RESULTS

OPHTHALMOSCOPY

The changes occurring in a resolving experimental vitreous clot have been documented by several workers[300,422,458]. The degree of vitreous opacity was staged according to clinical criteria described in Chapter 6. Following the injection of blood, a circumscribed clot occupied the central vitreous for 24-48 hours. From the third day, however, the vitreous became homogeneously opaque (Stage 1) (Fig.8.1). Numerous strands gradually developed throughout the vitreous with some increase in red reflex occurring about 4-5 weeks after injection (Stage 2). Fragmentation of the clot with fundus details visible (Stage 3) usually occurred between six and ten weeks. By twelve to sixteen weeks, most eyes had reached Stage 4 (clear central vitreous,residual small opacities) or Stage 5 (completely clear vitreous). However, in some rabbits, residual small opacities remained for up to thirty-two weeks after injection of blood, although very little solid material was obtained from the vitreous after ten weeks.

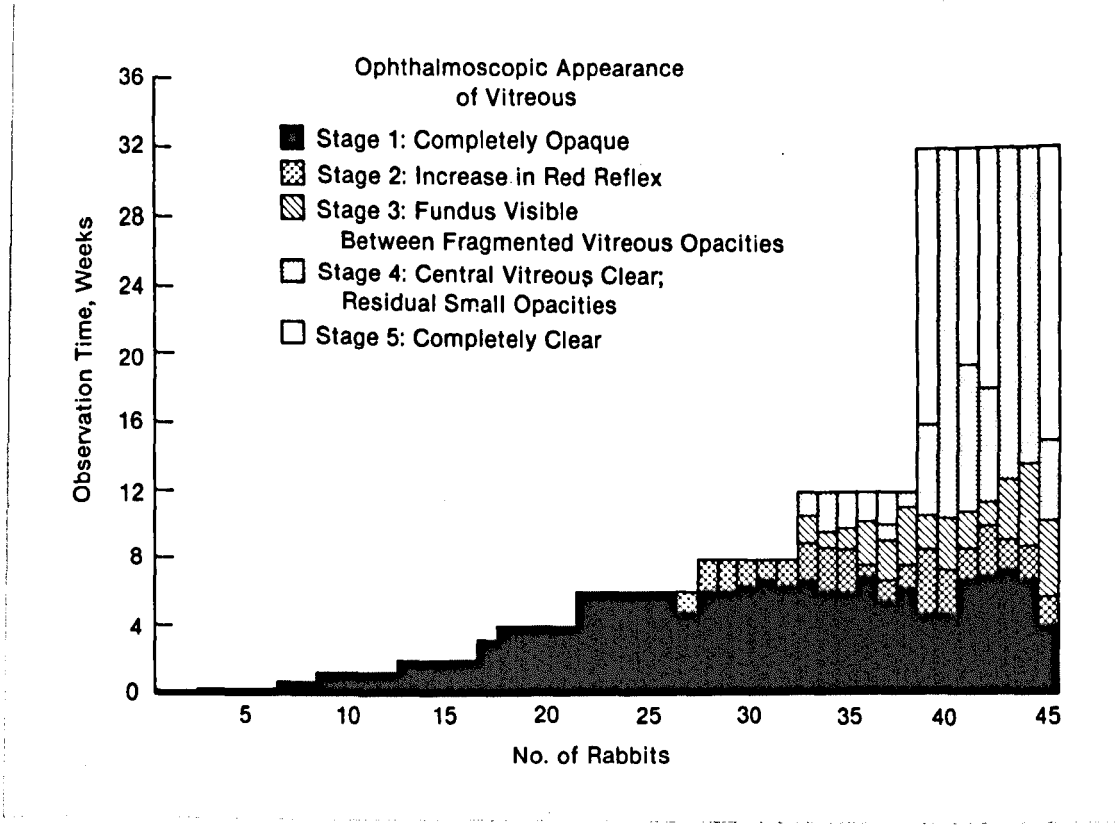


Figure 8.1 The degree of vitreous opacification was staged by clinical criteria as described in the figure. Each bar represents one animal, and the top of each bar corresponds to the time of sacrifice. It can be seen that by twelve weeks, most of the eyes had reached Stage 4 of clot resolution and by twelve to sixteen weeks, Stage 4-5.

FIBRIN ANALYSIS

Rabbit crosslinked fibrin (Fig. 8.2(a)) produced two prominent bands corresponding to the β chain and $\gamma\gamma$ dimer of the polymerised fibrin molecule (Lorand, 1972). The bands representing the α and γ monomers indicated that the fibrin was not fully crosslinked. Normal rabbit vitreous (Fig. 8.3(c)) contained no proteins with molecular weights corresponding to the fibrin chains, but several other soluble proteins were present[282]. Solubilised extracts from the solid vitreous clots of varying duration produced bands corresponding to the β monomer and $\gamma\gamma$ dimer chains, indicating the presence of strongly crosslinked fibrin within the vitreous for up to five weeks following clot formation (Fig. 8.2(b) to (f)). No fibrin was detected after this time (Fig. 8.2(g) and (h)). Several other lower molecular weight proteins were noted with two prominent bands appearing at molecular weights of 32,000 and 16,000 daltons (Fig. 8.2(x) and (y)). The lower band was thought to represent a monomer and the upper band a dimer of the constituent chains of the haemoglobin molecule, since two broad bands were observed in identical positions when rabbit haemoglobin was run on SDS polyacrylamide gel, as described in Chapter 6. It was not clear why the globin molecule was not fully depolymerised by the β-mercaptoethanol, but it may be related to the fact that the chain of rabbit globulin is not a single polypeptide but a mixture of closely related proteins[229]. It was considered that the presence of haemoglobin bands on gel electrophoresis of the vitreous extract probably represented protein released from intact red cells within the vitreous clot by the extraction procedure with 8M urea, since any free haemoglobin would have been removed during the preliminary washing procedure. These results indicated, therefore, that intact red cells were present for at least nine weeks

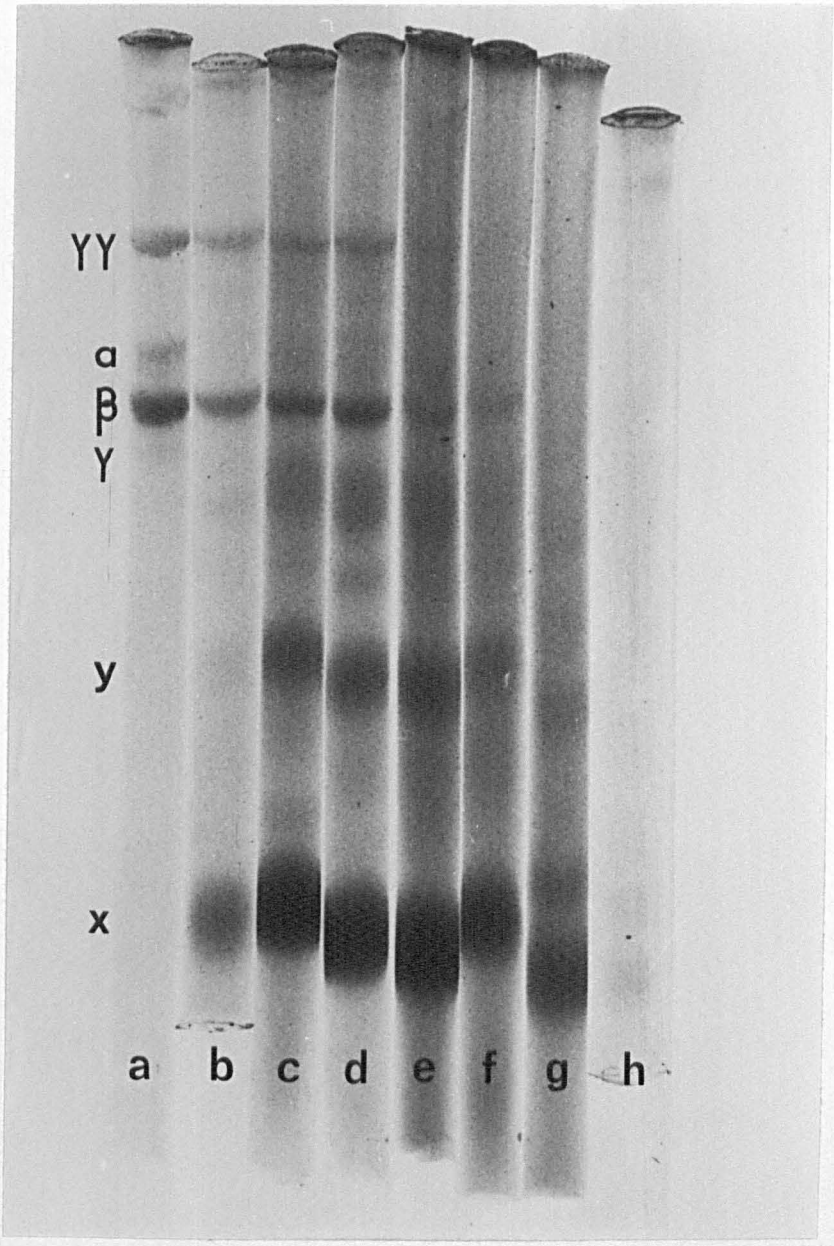


Figure 8.2 SDS-polyacrylamide gel electrophoresis of rabbit vitreous clots. (a) crosslinked rabbit fibrin, (b) - (h) rabbit vitreous clots solubilised with SDS and β-mercaptoethanol,(b) 2 days, (c) one week, (d) 2 weeks, (e) four weeks, (f) five weeks, (g) six weeks (h) nine weeks after injection of whole blood into the vitreous. α, β, γ and γγ dimer bands represent the constituent chains of the fibrin molecule. X, Y bands represent haemoglobin chains.

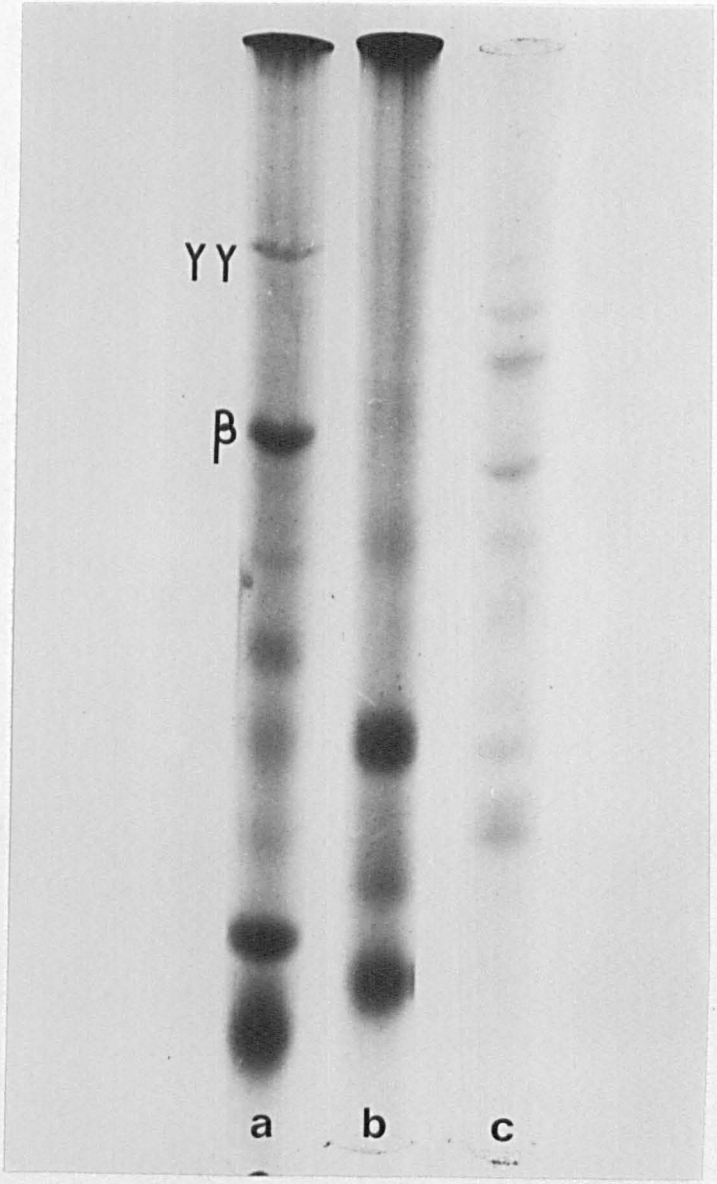


Figure 8.3 SDS polyacrylamide gel electrophoresis of rabbit vitreous clots. (a) control rabbit vitreous clot showing prominent β and $\gamma\gamma$ dimer fibrin chains, (b) streptokinase-plasminogen incubated vitreous clot showing absence of β and $\gamma\gamma$ dimer fibrin chains, (c) normal rabbit vitreous.

after clot induction.

Confirmation of the presence of fibrin with the vitreous clots was obtained by the disappearance of the β and γγ bands following incubation of the clot with streptokinase-plasminogen (Fig. 8.3). The quantity of fibrin estimated by gel scan spectrophotometry is shown in Table 8.1. Removal of the fibrin occurred progressively during the first six weeks of resolution.

DETECTION OF FDP

Precipitation rings in the radial immuno-diffusion plates surrounding the wells with supernatant vitreous were observed for up to six to eight weeks, following the injection of the blood (Fig. 8.4). Inconsistent results were obtained after this time, there generally being no FDP after two months. The concentration of FDP was estimated by interpolation from a standard curve of rabbit fibrinogen concentration vs square of ring diameter. Throughout the period of resolution of the clot, the level of FDP was low (Table 8.2). No FDP were found in normal rabbit vitreous (Fig. 8.4).

PLASMINOGEN ACTIVATOR ACTIVITY

Fibrinolytic activity of the vitreous was similar, following vitreous clot formation, to that of normal control samples, irrespective of the age of the clot (Table 8.3). Values were expressed in equivalent mean values of urokinase (Chapter 7).

TABLE 8.1

CONCENTRATION OF FIBRIN IN VITREOUS CLOTS

Time after induction of vitreous clot	Fibrin concentration mg/ml
2 days	0.105
4 days	0.130
1 week	0.150
2 weeks	0.038
4 weeks	0.021
6 weeks	nil

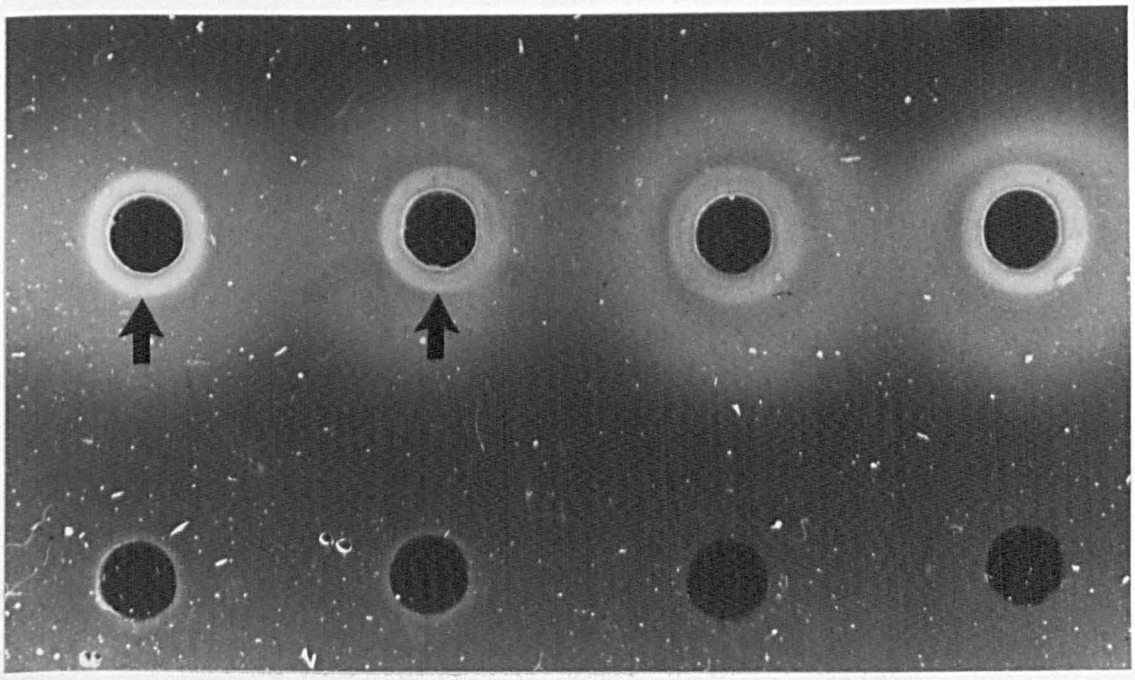


Figure 8.4 Radial immunodiffusion plates. Agar support contained 0.4% antirabbit fibrinogen serum. The supernatants from extracts of rabbit vitreous clots were placed in upper wells (top row). Immunoprecipitation lines were observed as shown (arrows). Diffuse haloes round each well were due to non-fibrin related proteins, mainly haemoglobin. Lower wells contained normal rabbit vitreous.

TABLE 8.2

FDP concentration in supernates from extracts of vitreous clots

Time after induction of vitreous clot	Sample No.	FDP µg/ml	
		Test	Control
2 days	1	10	nil
	2	18	nil
4 days	1	8	nil
	2	8	nil
8 days	1	5	nil
	2	5	nil
2 weeks	1	10	nil
	2	14	nil
4 weeks	1	8	nil
	2	8	nil
5 weeks	1	8	nil
	2	47	nil
6 weeks	1	nil	nil
8 weeks	1	nil	nil
	2	10	nil

TABLE 8.3

Plasminogen activator activity in vitreous extracts during vitreous clot resolution, expressed as equivalent Ploug units of urokinase

Sample No.	Test	Control	Time after induction of vitreous clot
1	0.40	0.37	2 days
2	0.41	0.39	2 weeks
3	0.43	0.46	4 weeks
4	0.00	0.42	4 weeks
5	0.41	0.41	8 weeks
6	0.41	0.38	8 weeks
7	0.40	0.35	32 weeks
8	0.37	0.35	32 weeks

DISCUSSION

These experiments have provided biochemical evidence for the presence of fibrin within experimental vitreous clots and have shown that it took five weeks for fibrin to be removed from rabbit vitreous following the injection of 0.2 ml autogenous whole blood. This time sequence is slow in comparison with other tissue deposits of fibrin[273] and it is probably related to the low levels of plasminogen activator activity within the vitreous (see Chapter 7). Current theories of thrombolysis (see Chapter 5) suggest that the rate of fibrin digestion is dependent on the plasminogen activator concentrations in the surrounding medium, and the present results would support this. In addition, a second factor in the delayed clearance of blood from the vitreous is suggested by the type of fibrin within a vitreous clot. Lorand and Jacobsen[298] have shown that crosslinked fibrin is more resistant to digestion by plasmin than fibrin monomer.* The experiments reported here clearly showed that fibrin within experimental vitreous clots was fully crosslinked, since there was no evidence of the α or γ monomer on SDS polyacrylamide gel electrophoresis (Fig. 8.1). Thus, the rate of intravitreal fibrinolysis would have been further reduced.

Also noteworthy was the constant level of plasminogen activator activity within the vitreous during clot resolution. Studies of fibrinolysis in other tissues have shown that there is an initial transient decrease in activator levels, followed by a rise in activity during the later stages of healing[514]. This secondary rise is related to the in-

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*FOOTNOTE: It should be noted, however, that these findings have recently been disputed[414].

growth of new vessels into healing tissues. The absence of this rise in activity in resolving vitreous clots would suggest an impairment of the healing process and, in particular, absence of ingrowing vessels into the vitreous. The morphological studies reported in Chapters 12-14 would support this concept.

Whatever the reasons for slow intravitreal fibrin digestion, it is clearly important to relate fibrinolysis to the whole of vitreous clot resolution. At least three processes are involved in the clearance of blood from the vitreous: haemolysis, fibrinolysis and phagocytosis by inflammatory cells. Horven[224] has shown by autoradiography that red cells cannot be reabsorbed intact from the vitreous into the general circulation, but that they must first be degraded and their breakdown products reabsorbed. Greer et al[196], using ^{51}Cr tagged red cells, confirmed that haemolysis is a rate-limiting step in vitreous clot resolution. The presence of FDP within the soluble component of the vitreous (Table 8.2) indicates that plasmin-mediated fibrinolysis also occurs in resolving vitreous clots and furthermore, the data suggest that haemolysis and fibrinolysis are simultaneous processes (Fig. 8.1), since the concentration of haemoglobin, which in the present study was a measure of residual intact red cells in the vitreous samples (see above), became progressively less. It is possible that the two processes are inter-related.

The role of inflammatory cells in fibrin removal from the vitreous is more difficult to define. It has been noted that leukocytes may clear fibrin either by fibrinolytic means, or by non-fibrinolytic protease digestion (Chapter 5). In addition, stimulation of the inflammatory response in the vitreous by short bouts of hyper-pyrexia[529] or by immunogenic or non-specific uveitis[44] has been reported to enhance the rate of clearance of vitreous blood. By contrast, suppression of

the inflammatory response with systemic ACTH retarded the rate of vitreous clot removal[458] and was associated with a reduced vitreous macrophage count. However, this latter finding could not be confirmed by Benson et al[44].

Maberly and Chisholm[300] confirmed the accelerated clearance of vitreous blood due to inflammation, in this case induced by the intravitreal injection of an inactivated fibrinolytic enzyme, streptokinase/streptodornase. However, these authors also observed that the active enzyme produced an even more rapid clearance, and suggested that clot lysis was due to the fibrinolytic effect of the enzyme rather than the inflammatory response alone. These results have been confirmed by our own group, using the fibrinolytic enzyme urokinase[148].

It is probable that both the inflammatory response and fibrinolysis, either separately or by interrelation, are relevant to the clearance of vitreous blood. The distinction between the two processes within the context of fibrin digestion may be artificial, but whatever the mechanism, fibrinolysis is an integral part of vitreous clot lysis.

SUMMARY

The process of fibrinolysis was studied in resolving vitreous clots in rabbits. The rate of fibrin degradation and removal was considered to be slow in comparison with other tissues, and the expected rise in tissue plasminogen activator activity during clot resolution was not observed. The relationship between haemolysis, fibrinolysis and the inflammatory response, and their importance to vitreous clot lysis, are discussed.

CHAPTER 9

THE EFFECT OF FIBRINOLYTIC INHIBITION ON THE RESOLUTION OF VITREOUS CLOTS

INTRODUCTION

The data presented in Chapters 7 and 8 suggest that the slow resolution of vitreous clots may be due, at least in part, to the low levels of plasminogen activator activity within the vitreous. To explore this possibility further, the experiments described in this chapter were undertaken. By using synthetic inhibitors of plasminogen activator, it was hoped that the role of fibrinolysis in vitreous clot removal could be isolated from other factors such as haemolysis and inflammatory cell activity which, it was assumed, would remain unaffected. It has been observed previously that such agents have little effect on leukocyte fibrin-splitting protease activity (see Chapter 5).

This approach to the study of wound healing processes is not new. Indeed, the concept of fibrinolysis in the regulation of tissue repair originates from ideas proposed by Fleisher and Loeb[140] at the beginning of this century (see Chapter 5). When suitable techniques became available, it was shown that fibrinolysis played a significant part in connective tissue repair[274] and that inhibition of fibrinolysis in rats led to disordered wound healing, with an exaggerated fibroblastic response[275]. More recently, it has been suggested that, in addition to local tissue activator, secretion of plasminogen activators by monocytes and macrophages may prove essential for wound healing to proceed normally[187]. Leibovich and Ross[294] showed that, although polymorphonuclear leukocytes migrated normally into skin wounds in monocyte-depleted guinea pigs, macrophages failed to accumulate in the wound area, with the result that there was a delay in the removal of fibrin, red cells and extracellular debris from the wound. In addition, fibroplasia and re-epithelialisation were retarded. Other studies have

shown a requirement for a normal polymorphonuclear response in fibrin clearance[434], and it is likely that the full expression of the inflammatory reaction is necessary for normal wound healing. Thus, although fibrinolysis is generally regarded as the major pathway for fibrin clearance from wounds (Chapter 5), the most recent evidence indicates that it may be difficult to separate fibrinolytic mechanisms from inflammatory cell activity in vivo. Indeed, the results of the present experiments suggest a close correlation between fibrinolysis and the inflammatory response in the clearance of blood clots from the vitreous.

MATERIALS AND METHODS

ANIMAL MODELS

New Zealand white rabbits, weighing 3-4 kgs each, received 0.5 g of 4 amino-methyl-cyclohexane-carbonic acid (AMCHA) in their drinking water daily. In preliminary experiments on six rabbits, this dose of AMCHA completely suppressed fibrinolytic activity of normal vitreous, when tested by the fibrin plate technique (Chapter 6). In the present experiments, 14 AMCHA-treated animals were tested, with 16 untreated controls.

INDUCTION OF VITREOUS CLOTS

This has been described in Chapter 6. The rate of clearance of the vitreous clot was monitored at weekly intervals by direct and indirect ophthalmoscopy, and the degree of vitreous opacity was graded on a scale of 1-5, as described (Chapter 6). Animals were sacrificed at the time intervals indicated in the Results and the vitreous was extracted immediately by the freezing technique (Chapter 3).

Preparation of the vitreous extract

Vitreous extracts were prepared for fibrin analysis on 7.5% polyacrylamide gel electrophoresis in sodium dodecyl sulphate, and detection of FDP as described in Chapter 6.

HISTOLOGY

Two eyes from AMCHA-treated rabbits were prepared for histology (see Chapter 11) after 36 weeks of observation. The globes were fixed in phosphate-buffered glutaraldehyde, embedded in paraffin and stained with haematoxylin and eosin, periodic acid schiff reagent, Van Gieson's stain and Mallory's phosphotungstic acid haematoxylin for the detection of fibrin.

RESULTS

OPHTHALMOSCOPY

The ophthalmoscopic appearances of vitreous clot resolution in the rabbit have been described (Chapter 8). In this study, all nine surviving control rabbits had reached Stage 4, and three had reached Stage 5, 12 weeks after the injection of blood into the vitreous (Fig. 9.1). In contrast, none of the eight AMCHA-treated rabbits, permitted to survive to twelve weeks, had progressed further than Stage 2, and after thirty weeks, five of the six surviving rabbits had reached only Stage 3.

HISTOLOGY

After 36 weeks of observation, histological examination of the

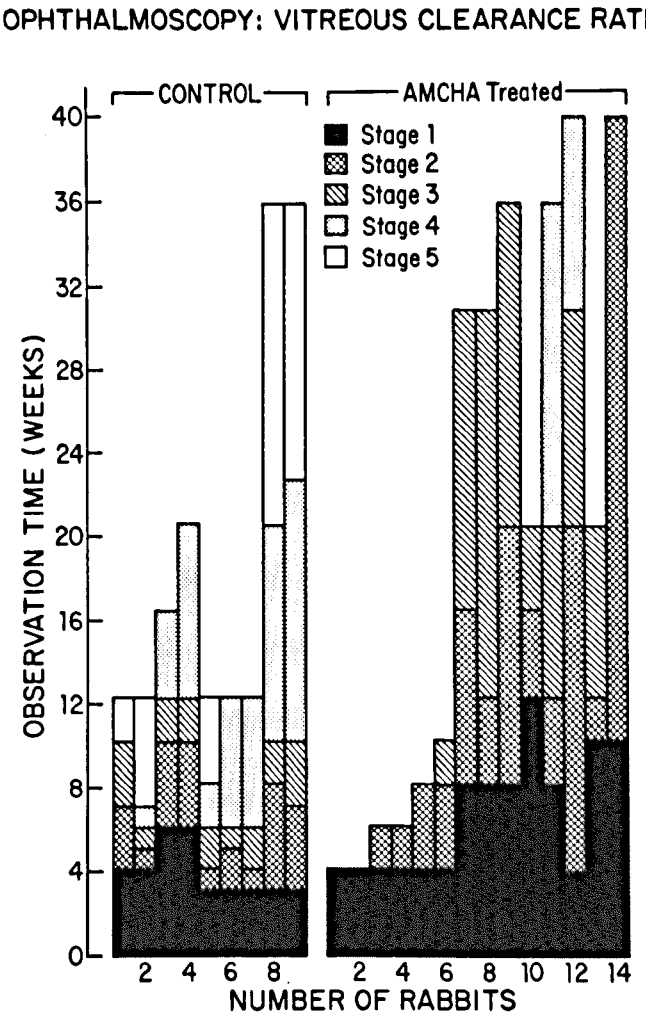

Figure 9.1 Ophthalmoscopy: Vitreous clearance rate. Each bar represents one rabbit. The various shades represent the stages of resolution of the haemorrhage (see Figure 8.1). The top of each bar indicates the time of killing of each rabbit. Control rabbits killed before 12 weeks are excluded from the figure. A significant reduction in the vitreous clearance rate in the AMCHA-treated group is shown.

vitreous opacities in two AMCHA-treated rabbits revealed the presence of clumps of intact red blood cells (Fig. 9.2) associated with a few large macrophages (Fig. 9.3) and surrounded by thin strands which were presumed to be vitreous collagen.

FIBRIN ANALYSIS

The presence of strongly cross-linked fibrin was observed within the vitreous of AMCHA-treated rabbits for up to six weeks, as shown by the β and $\gamma\gamma$ dimer bands (Fig. 9.4), with no evidence of the α or γ monomer chains. This contrasted with the previous observation of fibrin within untreated rabbit vitreous clots for up to five weeks only (Fig. 8.2). After six weeks, densitometric scanning of the gels from AMCHA-treated rabbits failed to detect fibrin within the vitreous, in spite of the presence of considerable clot debris at this stage (Table 9.1). However, prominent gel bands representing haemoglobin were present for periods up to forty weeks (Fig. 9.4), indicating the persistence of numerous intact red blood cells within the residual material. In untreated rabbits, these bands had mostly disappeared by twelve to sixteen weeks (Fig. 8.2).

DETECTION OF FDP

While FDP at low concentration (8 to 20 μg/ml) were detected in control rabbit vitreous, no FDP were detected in AMCHA-treated rabbits at any stage up to forty weeks (Table 9.2). The limit of sensitivity of the test was 1 μg/ml.

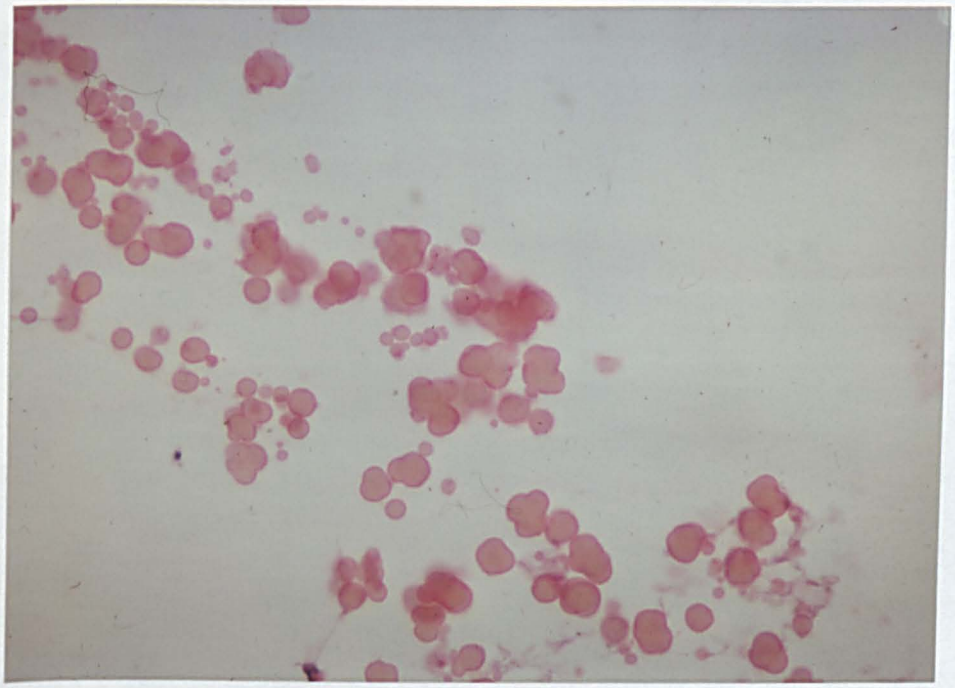

Figure 9.2 Histological appearance of vitreous opacity from AMCHA-treated rabbit after 36 weeks. Numerous intact red blood cells are present. Several abnormal forms of erythrocytes are also shown. X400.

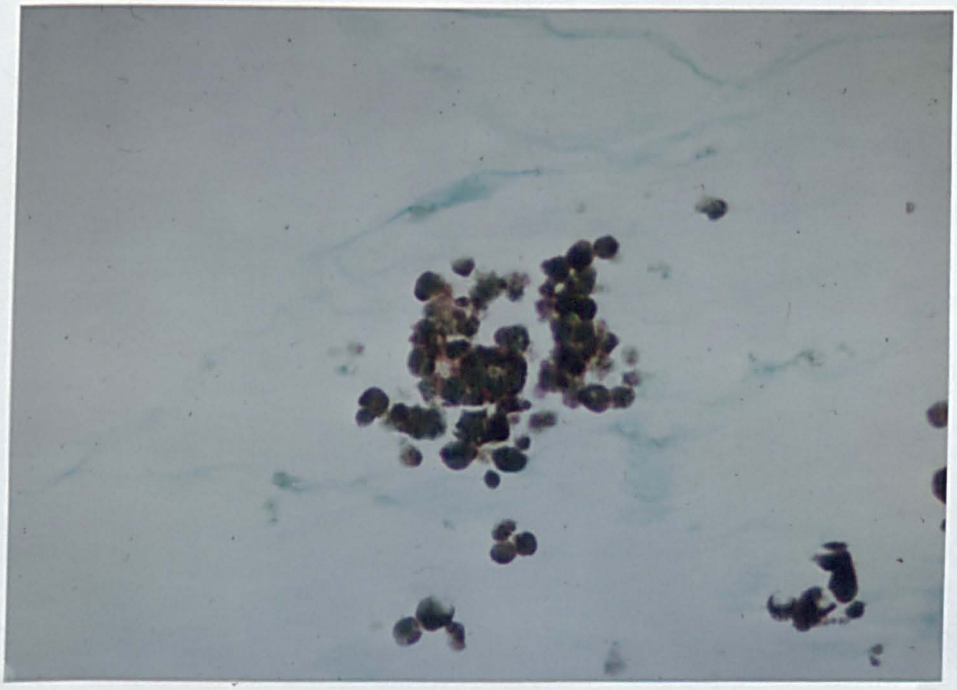

Figure 9.3 Histological appearance of vitreous opacity from AMCHA-treated rabbit after 36 weeks. A clump of large macrophages, some of which are multinucleate, is shown. X350.

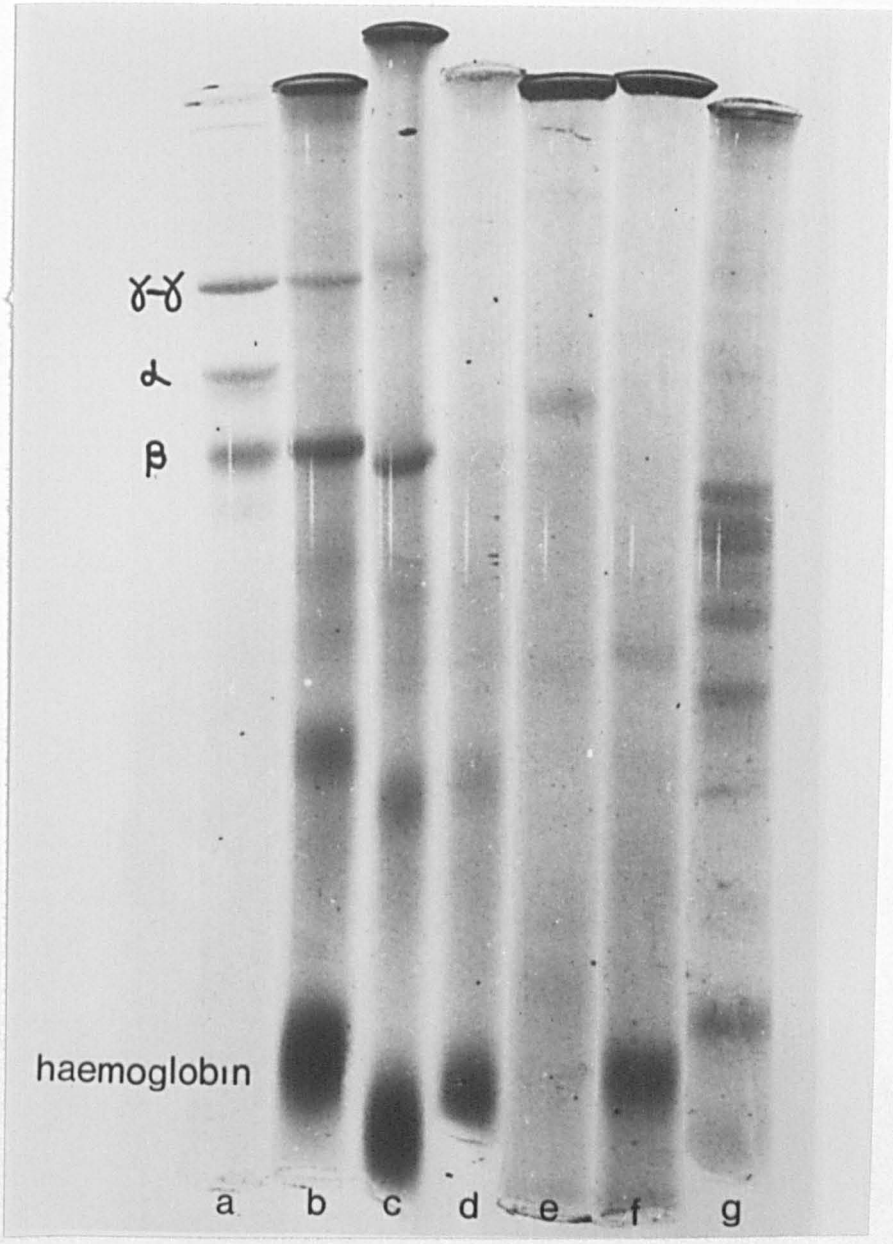

Figure 9.4 SDS-polyacrylamide gel electrophoresis of vitreous clot extracts from AMCHA-treated rabbits; (a) Typical cross-linked rabbit fibrin from plasma, showing prominent β and γ-γ dimer bands. Trace amounts of α chain present indicate incomplete cross-linking of fibrin. (b) and (c) Strongly cross-linked fibrin in vitreous clot extracts is shown by the presence of β monomer and γ-γ dimer bands. No α or γ monomer is present. (d) to (g) Absence of fibrin bands in vitreous after six weeks, (b) four weeks; (c) six weeks; (d) eight weeks; (e) ten weeks; (f) 20 weeks and (g) 40 weeks. Haemoglobin bands persist for up to 40 weeks of observation, indicating the presence of significant numbers of intact red blood cells for the duration of the experiment.

TABLE 9.1

Concentration of fibrin in extracts of vitreous clots

Time (wks) after induction of vitreous clot	Fibrin concentration mg/ml	
	Control (untreated)	AMCHA-treated
1	0.150	not tested
4	0.010	0.059
6	nil	0.040
8	nil	nil
12	nil	nil
20	nil	nil
40	nil	nil

TABLE 9.2

FDP concentration in supernates from vitreous extracts during vitreous clot resolution

Time (wks) after induction of vitreous clot	FDP concentration (mg/ml) Control (untreated)	AMCHA-treated
1	20	nil
4	8	nil
6	14	nil
8	10	nil
12	8	nil
20	nil	nil
40	nil	nil

DISCUSSION

This study has shown that inhibition of rabbit vitreous fibrinolytic activity produced a slight, but probably not significant delay in the removal of fibrin from the vitreous (Table 9.1). However, the absence of FDP within the supernatant vitreous extract from the AMCHA-treated rabbits confirmed the inhibition of plasmin-mediated fibrinolysis and suggested that the removal of the vitreous clot fibrin was achieved by other means, such as direct macrophage activity[288]. Paradoxically, there was a significant delay in the rate of clearance of the vitreous opacity in the AMCHA-treated rabbits as compared to the untreated control (Fig. 9.1). Both histological (Fig. 9.1) and biochemical (Fig. 9.4) analyses of the vitreous showed that a large proportion of this visible blood mass was composed of intact red blood cells. Since red blood cells within the vitreous are generally removed by macrophage endocytosis[194,224,369], the persistence of vitreous red blood cells suggests that either the phagocytic ability of the incoming macrophages was impaired, or that the cellular response within the vitreous was diminished. Many endogenous agents enhance leukocyte migration towards foci of inflammation[555] including FDP[34,324,485]. Hence, the absence of FDP in the AMCHA-treated rabbits may have contributed to a reduced cellular response within the vitreous. Indeed, the poor resolution of vitreous haemorrhages in general (see Chapter 1) may be caused by a low level of chemotactic factors. In vitro studies have shown that maximum chemotactic effect is elicited by FDP at a concentration of 100 to 300 $\mu g/ml$[485] In untreated rabbit vitreous clots, the FDP levels were considerably lower (8–20 $\mu g/ml$; Table 9.2). FDP concentration within the range 100–400 $\mu g/ml$ have been observed in rabbit vitreous clots treated with the plasminogen activator, urokinase, and were associated with a

significantly more rapid rate of vitreous clearance[148]. Therefore, indirect evidence suggests that low levels of fibrinolytic activity in the vitreous may be partly responsible for the poor rate of clearance of the vitreous clot, not only by prolonging fibrin removal, but also by failing to provide an adequate chemotactic stimulus. Such evidence may reflect the interdependence of fibrinolysis and the inflammatory response, which has already been emphasised.

SUMMARY

The effect of fibrinolytic inhibition on the resolution of vitreous clots in rabbit eyes was studied. Although there was only slight delay in the removal of intravitreal fibrin in AMCHA-treated rabbits as compared to controls, there was a significant reduction in the rate of red cell clearance. It is suggested that the absence of plasmin-mediated chemotactic factors led to an impaired phagocytic cellular response in the treated animals. Similar mechanisms may be of importance in the non-resolution of human vitreous haemorrhage.

PART 4

THE PATHOLOGY OF VITREOUS HAEMORRHAGE

CHAPTER 10

REVIEW OF THE LITERATURE ON PATHOLOGICAL CHANGES WITHIN THE VITREOUS AFTER HAEMORRHAGE

INTRODUCTION

It is a long-held view that fibrous organisation takes place as part of the healing mechanism within non-resorbing vitreous clots, particularly if some "irritative" factor is present[126] (see Chapter 1), and indeed the pathological changes that occur within a vitreous haemorrhage have been likened to those that occur within a healing wound. Experimental studies of skin wounds[52, 379] have identified three phases in the evolution of the healing process: a phase of acute inflammation where an exudate of polymorphonuclear leukocytes and fibrin develops, a phase of demolition where tissue cells and inflammatory cells undergo autolysis and macrophages remove the debris, and a phase of granulation tissue formation where there is proliferation and migration of surrounding connective tissue elements. It is outside the scope of this thesis to review current knowledge of the wound healing process since this now encompasses several large areas of intensive scientific interest such as cell migration, mediators of inflammation and endothelial cell proliferation. However, the simple concept described above is useful as a framework for the discussion of the process of blood reabsorption from the vitreous.

Early investigators realised that little information concerning the pathophysiology of vitreous haemorrhage resolution could be gained from the study of clinico-pathological material, since in most cases, enucleated human eyes with vitreous haemorrhages are complicated by other changes, such as intractable glaucoma or severe perforating injuries. Experimental methods for the study of vitreous heamorrhage reabsorption, therefore, have been adopted, the first by Probsting[404] and, almost exclusively, the animal model has been the rabbit. Several

aspects of this process have been investigated, including the inflammatory cell response, red cell degradation and removal, fibrin degradation, and fibroplasia. However, as Cibis[88] has observed, numerous technical problems have been encountered with the fixation and structural preservation of delicate vitreous tissue, and therefore non-histological techniques have often been employed. A brief review of previous work in this field is described here.

THE INFLAMMATORY CELL RESPONSE

This has been studied on several occasions since 1892[83,159,160,194,224,253,369,404,458]. Gray[194] observed that in the first few hours after injection of blood into the vitreous, very little reaction had occurred. After twenty-four hours, inflammatory cells were seen invading the vitreous cavity, but there was a predominance of mononuclear cells, as compared to the marked polymorphonuclear cell reaction to injections of bacteria or dye into the vitreous. In the later stages, large macrophages were observed which were engaged in phagocytosis of red cells and detritus. Most of the other studies refer to the later stages of vitreous haemorrhage reabsorption, and in all cases, the presence of very large macrophages within the clot debris has been noted. Horven[224] drew a similarity between these cells and the compound granular corpuscles of the brain, and was thus in agreement with Wolter[568], who suggested, on morphological evidence alone, that such cells originated from microglial cells within the retina.

HAEMOLYSIS OR RED CELL DEGRADATION

Haemolysis has been considered an important stage in vitreous

haemorrhage reabsorption, and it can be regarded as the phase of demolition in the wound healing process. Radioactive tracer studies[63] suggested that haemolysis was a rate-limiting step in vitreous clot resolution, although the experimental method was later questioned[44]. Phagocytosis of red cells has been observed in all histological studies to date, and Horven[224] showed that red cells in the vitreous, unlike those in the aqueous, underwent lysis before they were removed from the eye. Regnault[422] further demonstrated that haemoglobin was fully degraded to bilirubin and globin within the vitreous. This process probably occurs within vitreous macrophages.

FIBRIN REMOVAL

The degradation and removal of fibrin from the vitreous during vitreous clot lysis has rarely been studied. Occasional histological studies have testified to the presence of fibrin within experimental vitreous clots, but fibrinolytic mechanisms per se have not been investigated. In Part 3 of this thesis, it has been shown that vitreous fibrinolytic activity is of a low order, and it has been suggested that this may be a factor in the delayed resolution of vitreous clots. The relationship between fibrinolysis and the inflammatory response during vitreous clot resolution has also been discussed (Chapter 9).

FIBROUS ORGANISATION OF VITREOUS CLOTS

The commonly-held belief that fibrous organisation occurs within non-resolving vitreous clots, is the counterpart of phase three in the wound healing process. However, it has not been clearly shown that

blood within the vitreous provides a sufficient stimulus for fibroblastic activity from the surrounding ocular structures. Most reports which described vitreous fibrosis after haemorrhage, concerned clinico-pathological case studies on severely injured eyes or on eyes that were associated with retinal disease such as diabetic neovascular proliferation or retinal vasculitis[141,255,267,287,330,355,401,441,513,528,546]. Experimental attempts in animals to stimulate fibroblastic activity within the vitreous by injections of blood have had a varied response[83,89,159,369,571]. Under these circumstances, vitreous fibrosis was normally associated with a traumatic injection procedure, involving perforation of the globe through the retinal layers with aspiration of vitreous[159,404], or there was an associated retinal detachment[83,159,369]. In all cases where fibrous tissue formation was reported, the fibrosis took the form of an epiretinal membrane, and in no instance was fibroblastic activity found within the blood clot. Moreover, the incidence of fibrosis in the vitreous was low after a single blood injection. Only when cultured fibroblasts from the skin were injected intravitreally was a high rate (70%) of intravitreal fibrosis achieved[3]. In contrast, several other workers have failed to report the presence of fibrosis after injection of blood into the vitreous[194,196,300,422,458,529], and indeed on occasion have drawn attention to this fact[194,287,369,476]. Gray[194] noted that any fibroblastic activity in his specimens was associated with the injection wound in the scleral/choroidal coat of the eye, and that neither homologous nor heterologous blood deposits in the vitreous showed any tendency to fibroblastic or glial cell invasion.

CONCLUSION

The pathological changes occurring within a resolving vitreous clot have been studied experimentally on several occasions, but considerable controversy surrounds the sequence of events which typically occur. This is due possibly to the introduction of complicating factors, such as severe ocular trauma, in several of the studies. Little is known concerning the early stages of the inflammatory response, and the question of whether fibrous organisation occurs within uncomplicated vitreous clots remains unanswered. In addition, the process of intravitreal haemolysis merits further morphological study. The following chapters describe the histological and ultrastructural changes within experimental vitreous clots, and the results are discussed in relation to the above points.

CHAPTER 11

MORPHOLOGY OF VITREOUS BLOOD CLOTS: MATERIALS AND METHODS

INTRODUCTION

This chapter contains details of the techniques used for histological and ultrastructural studies of experimental vitreous clots. The difficulties surrounding the preparation and fixation of delicate vitreous tissue have been referred to in Chapter 10. In addition, it is common knowledge that routine sectioning of whole fresh rabbit eyes in the sagittal plane causes considerable tissue distortion due to differences in tissue hardness and shape between the lens and remaining ocular structures. For histological studies, therefore, a freezing technique was used which avoided gross tissue disturbance. Artefacts due to ice-crystal formation were not observed, and satisfactory tissue sections for light microscopy were obtained. For electron-microscopy, the eye was sectioned after fixation in a coronal plane which was chosen to avoid intravitreal structures. Pieces of tissue from the vitreous were then dissected under magnification.

ANIMAL MODEL

New Zealand white rabbits were used as before (Chapter 6). The rabbit is not the ideal model for comparison with the human in the study of vitreous pathology, due to anatomical and biochemical differences (see Chapter 1). Rabbit vitreous contains more collagen and less hyaluronic acid than human vitreous, and the relative vitreous volume is considerably smaller. However, it was considered suitable for the purposes of this study, since the nature of the vitreous was similar in both species (i.e., a gel) and since the anatomic relationships, if not the proportions, also bore a reasonable resemblance.

INDUCTION OF VITREOUS CLOT

This has been described in Chapter 6.

PRIMARY FIXATION

Immediately after enucleation, the eyes were immersion fixed in 2-4% phosphate buffered glutaraldehyde (Sorensen's phosphate buffer, 0.1M, pH 7.2-7.4) for 12-24 hours. Selected eyes for transmission electron microscopy were fixed in glutaraldehyde containing a 3% solution of 0.2M sodium cacodylate buffer (pH 7.2-7.4).

MACROSCOPIC EXAMINATION

PREPARATION OF THE TISSUES

A freezing technique was used to avoid disturbing topographical relationships within the eye. The eyes were placed in polythene bags and immersed in a solution of carbon dioxide snow in acetone for 60 seconds. The frozen globes were sectioned in a saggital plane through the pupil-optic nerve axis in most cases, using a Thiersch skin graft knife. A few eyes were sectioned in the coronal plane, 5 mm behind the lens. The frozen half-globes were placed on specially designed chucks and immersed in phosphate buffer. They were then allowed to thaw, and photographed.

LIGHT MICROSCOPY

EMBEDDING PROCEDURE

Care was taken to ensure that the delicate vitreous tissue was

undisturbed during the tissue dehydration through graded alcohols and embedding procedures. The half-globes were embedded in Paraplast wax at a temperature of 75°C for 2-4 hours and 6-8 μ sections taken.

TISSUE STAINING

The following tissue stains were used:

1. Haematoxylin and eosin.

2. Mallory's phosphotungstic acid haematoxylin (PTAH) for the demonstration of fibrin. In later experiments, Martius Scarlet Blue (MSB) was also used to detect fibrin.

3. Masson for the demonstration of collagen.

4. Modified Mallory trichrome for the demonstration of platelets[76].

5. Prussian blue for iron staining.

6. Periodic acid-Schiff base to stain for carbohydrate.

7. Elastic Van Giesen.

Conventional staining procedures were followed.

TRANSMISSION ELECTRON MICROSCOPY

The above methods of preparing the tissues for gross and histological examination were considered unsuitable for electron microscopy due to possible artefacts related to the freezing technique. Electron microscopic studies were therefore performed on a separate group of rabbit eyes.

TISSUE DISSECTION AND PREPARATION

After twenty-four hours in the primary fixation fluid, the eyes

were placed cornea down in eye cups. The posterior segment of the globe was removed with a Thiersch skin graft blade, 4 mm from the posterior pole of the eye. This plane of section was posterior to the vitreous clot, and thus did not interfere with the topographical relationships. Small segments of tissue were dissected from the vitreous clot and membranes under magnification, using microsurgical scissors (Vannas) and forceps.

SECTION PROCEDURE AND VIEWING OF TISSUE

The tissue blocks were washed in phosphate buffer for at least two hours, after which they were post-fixed in 1% osmium tetroxide (TAAB laboratories) in cacodylate buffer. The tissue was then re-washed in buffer, dehydrated through graded alcohols and embedded in Araldite (TAAB laboratories). Sections of tissue were cut using an LKB Ultratome III. Thick sections (1.0, 1.5, 2.0 μm) were stained with Toluidine blue and ultrathin sections (500-800A$^{\circ}$ with uranyl acetate and lead citrate. They were mounted on copper grids (100 and 200 mesh) and viewed with a Phillips 301 electron microscope.

ACID PHOSPHATE STAINING PROCEDURE

Selected tissue segments were treated by a modification of the Gomori method for the demonstration of acid phosphatase activity, as described by Miller and Palade[335]. Sections were rinsed for twenty minutes to two hours in one to two changes of cold Na-cacodylate buffer (pH 7.4) and finally incubated for 15 minutes in fresh Gomori medium. This was composed of 0.12 g $Pb(NO_3)_2$ in 100 ml 0.05M Na acetate buffer, pH 5.0, containing 7.5% 0.22M sucrose to which 10 ml of 3% Na-β-glycerophosphate was slowly added. Before use, the mixture was warmed at 60°C for one hour, cooled to room temperature and

filtered to remove the slight precipitate which usually developed. After incubation, the sections were rinsed twice for one minute in cold 0.05M Na acetate buffer (pH 5.0) containing 7.5% sucrose and 2-4% glutaraldehyde, with a short rinse in 2% acetic acid in between the two rinses of acetate-buffered glutaraldehyde. Controls from which the substrate was omitted were also run.

SCANNING ELECTRON MICROSCOPY

TISSUE DISSECTION

The remainder of globe was partially sectioned in several meridional planes and each scleral flap was slightly stretched to expose the contents of the globe still further, but not to such a degree that rupture of the vitreous membranes occurred. In selected eyes, further dissection of vitreous tissue was performed to reveal the surface of specific structures such as the fibrin clot.

TISSUE DRYING

The tissue was freeze-dried using a procedure modified from that described by Boyde and Wood[61]. The specimens were rinsed in distilled water and the superficial fluid removed on lint-free absorbent paper. They were then quenched in liquid Arcton[12] (boiling point $-39^{\circ}C$, melting point $-155^{\circ}C$) which had melted after first being solidified over liquid nitrogen. The boat was placed on a cold plate in the vacuum chamber of a Balzars micro BA3 freeze drying apparatus. Drying took place after eight hours at a pressure of 1×10^{-5} Torr.

COATING AND VIEWING OF THE DRIED TISSUE

The dried specimens were mounted on standard viewing stubs with silver conducting paint (Acheson Colloids Co.) and coated with gold in a Polaron Sputter Coater E 5,000. The specimens were examined in a Cambridge Sterioscan S600 at accelerating voltages between 7.5 and 25 KV. The machine was capable of an optimum resolution between 200 and 300^{o}A.

CHAPTER 12

MORPHOLOGICAL STUDY OF VITREOUS CLOT LYSIS:

GROSS AND HISTOLOGICAL APPEARANCES

INTRODUCTION

Clinical and experimental studies on the pathology of vitreous haemorrhage have failed to establish a cause for the poor resolution of vitreous clots. This is partly the result of conflict of opinion concerning the natural history of vitreous clot lysis (see Chapter 10). Several aspects of the resolution process have been investigated in recent years, including haemolysis[63, 196], phagocytosis[224], membrane formation[278,490], connective tissue formation[159,458,571], fibrinolysis (see Part 2) and the ocular toxicity of blood[422] and iron compounds[89]. However, many years have elapsed since the last detailed morphological study of vitreous clot lysis[194]. Although Regnault[422] was the first to provide documentary evidence of the gross morphology of vitreous clot lysis, his illustrations were difficult to interpret and, in addition, there was no corresponding histological study. The present study was therefore undertaken to review the macroscopic and histological appearances of experimental vitreous haemorrhages.

MATERIALS AND METHODS

Forty-five New Zealand white rabbits were used in this study. The method for induction of vitreous clots is described in Chapter 6. The clearing of the blood from the vitreous was monitored weekly by direct and indirect ophthalmoscopy for periods up to 32 weeks after injection. The methods of macroscopic and histological examination of the eyes are described in Chapter 11.

RESULTS

OPHTHALMOSCOPIC OBSERVATIONS

The ophthalmoscopic observations of experimental vitreous clot lysis are described in Chapter 8 (see Fig. 8.1) in which a numerical scoring system was used to record the degree of vitreous opacity. The findings are in agreement with most other studies[63,159,196,300,369,458,565]. Substantial changes in the vitreous opacity began about four weeks after induction of the haemorrhage, and by 12 to 16 weeks, most of the surviving rabbits had reached Stage 4 or 5. However, in some rabbits residual opacities remained for up to 32 weeks, when the experiment was concluded.

MACROSCOPIC APPEARANCES

Twenty-four hours after injection of blood into the vitreous, a large circumscribed clot was observed (Fig. 12.1a), with smaller clotted masses delineating the needle track through the gel. No diffusion of blood had occurred, and the surrounding vitreous gel was uninvolved. After one week, however, several changes had occurred (Fig. 12.1b). Considerable haemolysis had led to uniform dark discoloration of the vitreous cavity, presumably because of released haemoglobin pigment. The original blood clot now appeared as a pale globular mass that occupied the central vitreous. In addition, detachment of the solid vitreous gel from the retina had occurred with the formation of many veil-like sheets of "collapsed" vitreous surrounding the central clot like a capsule (Fig. 12.1b, arrows). Numerous connections remained between this pseudocapsule and areas where vitreous

Legends for Figure 12.1

Time course of blood clearance from vitreous of rabbit: gross pathology

(a) 24-hour haemorrhage. Circumscribed dark clot is shown in midvitreous space behind lens. Less dense clotted blood is also present as thin strands delineating the needle track through the gel from the injection site. Surrounding vitreous gel is not affected.

(b) One-week haemorrhage. Vitreous cavity is diffusely, dark stained, probably due to red cell lysis. Original clot has become pale and globular. Extensive destruction of the vitreous gel has occurred, including detachment of the gel from the posterior retinal surface and formation of brown-stained fine sheets of tissue that appear to be surrounding the main clot mass.

(c) Two-week haemorrhage. Vitreous is less darkly stained. Coalescence of sheets of tissue has occurred to form prominent single sheet or "vitreous membrane" extending across the vitreous cavity and encapsulating the clot that is now firmly adherent to the posterior lens surface. Detachment of the vitreous has continued, leaving a clear space behind the vitreous membrane.

(d) Four-week haemorrhage. Almost complete detachment of the vitreous gel has occurred; only at optic nerve head does attachment between vitreous fibres and the posterior wall of the globe remain. Coloration of the vitreous is diffusely yellow-brown.

(e) High-power view (d) showing dense pseudocapsule around the clot.

(f) Six-week haemorrhage. Clot has disintegrated, leaving remnants of pseudocapsule surrounded by friable blood deposits. The

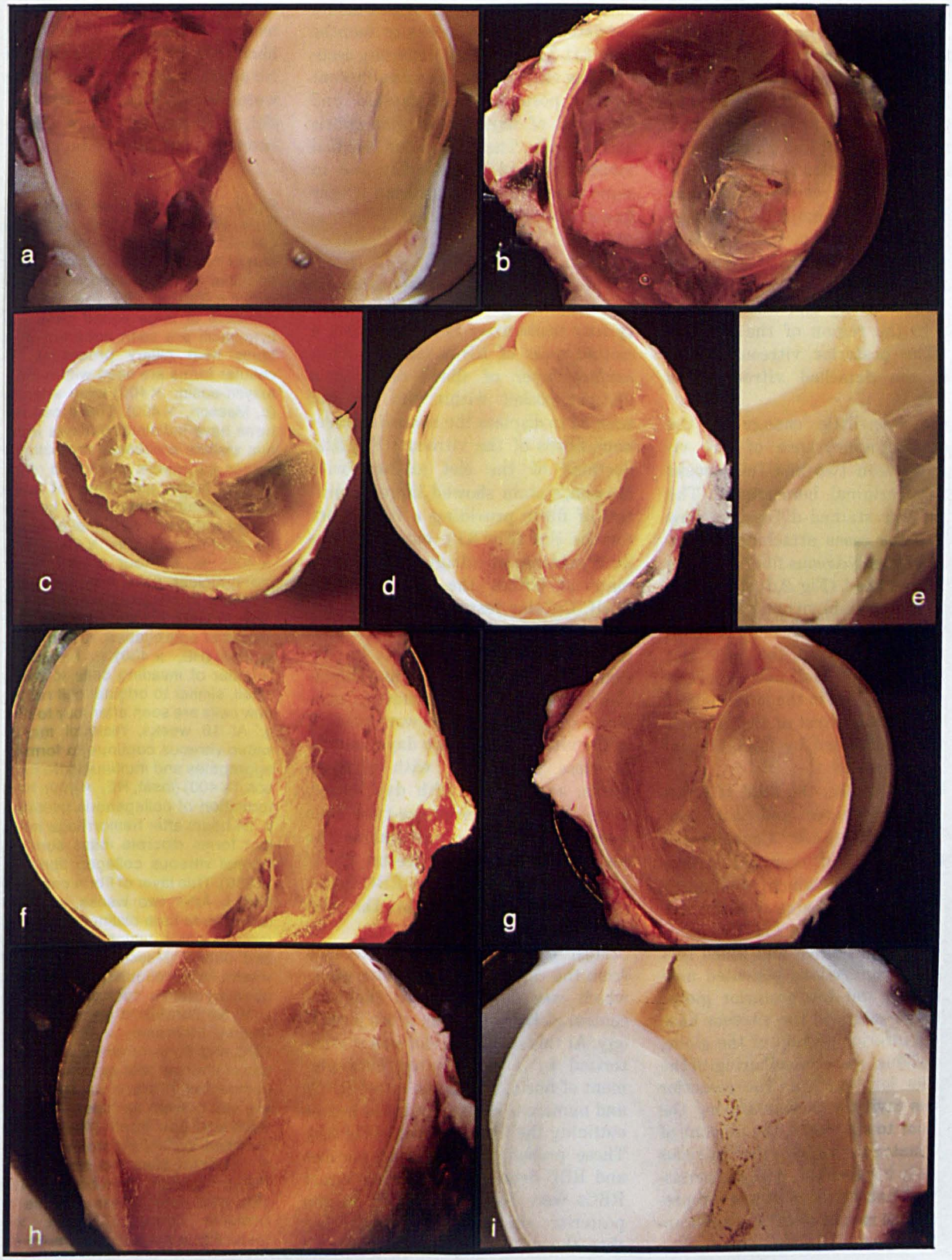

Figure 12.1.

vitreous is now fully detached and solid material is in close apposition to the lens surface. Posterior vitreous is clear and the vitreous fluid is xanthochromic.

(g) Eight-week haemorrhage. Continued clearing of the blood deposits has resulted in some solid material remaining on lens surface and in the vitreous base. This represents remnants of the pseudocapsule plus associated non-resorbed blood.

(h) Twelve-week haemorrhage. The vitreous is free of haematogenous material but the gel structure is considerably disturbed, as shown by its poor transparency.

(i) Sixteen-week haemorrhage. Small residual blood deposits are seen at this stage within the vitreous, especially in the vitreous base and adherent to the posterior lens surface. Central vitreous clear.

detachment was incomplete, e.g. the optic nerve and the vitreous base.

After two weeks, further changes had occurred (Fig. 12.1c). The colour of the vitreous had changed from dark red to yellow-brown. The original clot had become smaller and less globular, and was in close apposition to the posterior lens surface. Vitreous detachment was now almost complete, and the blood clot was completely enclosed within a dense sheet of collapsed vitreous that often extended across the vitreous cavity and was attached firmly to the region of the vitreous base (Fig. 12.1c, arrows); the posterior vitreous cavity behind the detached vitreous was clear.

There was little change in the macroscopic appearance of the vitreous between two and four weeks after the original haemorrhage. The vitreous was stained diffusely yellow and in some cases attachments persisted between vitreous fibres and the optic nerve head (Fig. 12.1d). However, the remaining solid vitreous was fully detached and a well-developed pseudocapsule surrounded the pale clot (Fig. 12.1e). The clot itself now generally occupied a position in the lower half of the globe and the rest of the vitreous cavity was occupied by fluid. No gel vitreous remained.

After six weeks, the fluid vitreous had become clearer and was now lightly xanthochromic. Considerable disintegration had occurred within the original clot, which was much reduced in size and appeared fibrillar, with cystic spaces (Fig. 12.1f). The pseudocapsule was friable and much less dense. No connections persisted between it and the posterior globe. Clot debris occupied the vitreous base and the inferior portion of the globe, with residual material adhering to the posterior lens surface. The posterior vitreous cavity was clear. During the next four to six weeks, the amount of solid material within the vitreous gradually became less, with the persistence of fine

vitreous strands representing the remains of the pseudocapsule (Fig. 12.1g).

After 12 to 16 weeks, the vitreous space was clear in half of the cases studied (Fig. 12.1h). However, in several eyes, the removal of blood was incomplete, and small amounts of clot debris persisted for many weeks or months (Fig. 12.1i). This material generally occupied the vitreous base or was adherent to the posterior lens capsule and consisted of dark brown clot debris and strands. The amount of residual material varied considerably, and in some cases was no more than a very fine strand.

HISTOLOGY

The histological appearances closely paralleled the gross pathology. Twenty-four hours after blood injection, a clotted mass of red blood cells (RBCs) coated with a surface layer of fibrin occupied a distinct pocket within the gel and seemed to displace the normal collagenous fibrils of the vitreous (Fig. 12.2k). Sections of the clot stained with Masson's stain, showed large quantities of fibrin providing a framework for the clot (Fig. 12.2k), but there was little evidence of collagenous-staining material within the clot itself. The cellular response at this stage was minimal, occasional single round cells being observed (Fig. 12.2a). In particular, there were few polymorphonuclear (PMN) cells. However, increasing numbers of cells were present in the vitreous after two days, usually in close association with vitreous fibrils, and by the fifth day larger, more definite macrophage-type cells were observed, aggregated along the condensed, detached vitreous fibrils and in close proximity to RBC material that was trapped within the solid vitreous (Fig. 12.2b).

Legend for Figure 12.2

Time course of blood clearance from the vitreous: histology

(a) through (g) cellular response.

(a) 24 hours after induction of haemorrhage occasional small mononuclear cells are seen within the clot (Masson, x400).

(b) By five days, greater numbers of large round cells with clear cytoplasm are seen adherent to vitreous fibrils near the ciliary processes (H and E, x400).

(c) Ten day clot. Unusual stellate cell forms are seen within centre of fibrin clot, but significant inflammatory cell invasion of the clot has not occurred (Masson, x400).

(d) Three-week haemorrhage, posterior vitreous. Large quantities of amorphous darkly-staining material (PTAH, x400). Inset, cell free nuclei associated with amorphous debris (H and E, x400).

(e) Four-week clot. Fresh, small mononuclear cells invading clot structure (H and E, x400).

(f) Four week haemorrhage, posterior vitreous. Clump of large, effete macrophages associated with free, intact red cells. Macrophages are in various stages of aggregation, apparent fusion and degeneration. Total cellular infiltrate remains scanty. Few young monocytes observed after four to five weeks (Masson, x400).

(g) Sixteen week haemorrhage. Residual vitreous macrophages form large cell aggregates and multinucleate giant cells (Masson, x400; inset: H and E, x400).

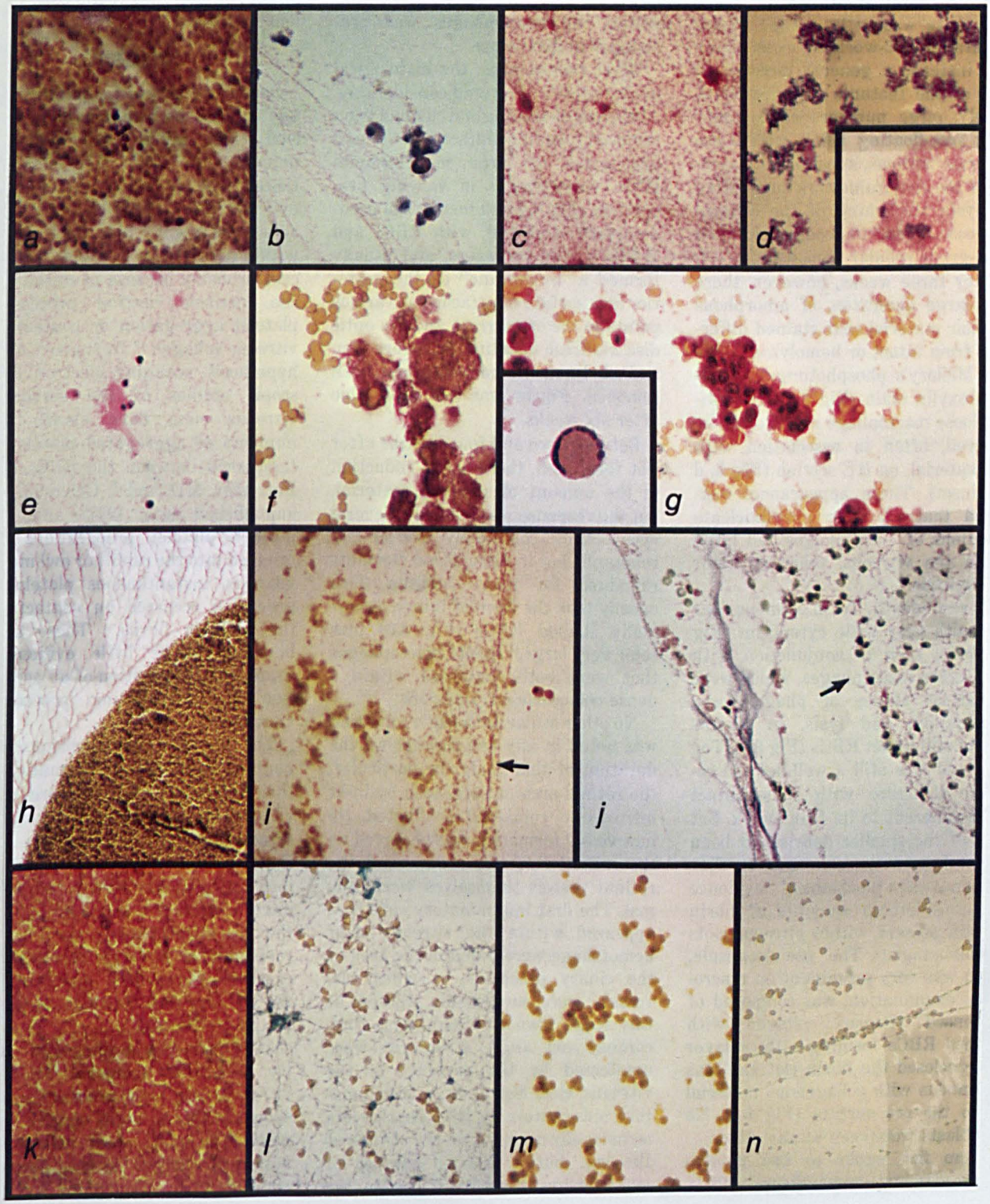

Figure 12.2.

(h) through (j)

(h) 24 hour haemorrhage. Collagen fibrils coalesce to form a distinct layer round the clot (arrow), within which are trapped red cells and debris (Masson, x250).

(i) Two week haemorrhage. Collagen fibrils coalesce to form a distinct layer round the clot (arrow) within which are trapped red cells and debris (Masson, x250).

(j) Four week haemorrhage. Loose reticular clot (arrow) is enveloped by dense collagenous pseudocapsule. No fibroblasts are noted (modified Mallory trichrome, x400).

(k) through (n)

(k) 24 hour haemorrhage. Large quantities of fibrin appear as pink fibres between compacted red cells (Masson, x400).

(l) Four week haemorrhage. Reticular fibrin strands within clot (modified Mallory trichrome, x250). No fibrin was seen after this stage.

(m) Four week haemorrhage. Free red cells in posterior vitreous cavity (Masson, x400).

(n) Twenty four week haemorrhage. Intact red cells adherent to vitreous fibrils. Pykuosis and anisocytosis are evident in several cells.

Most of the RBCs had been released from the central clot after one week, which, indeed, was suggested by the appearance of the clot as a pale globular mass on gross pathology (Fig. 12.1b). At this stage, the clot was characterised by a complicated entanglement of fibrin, with some intact RBCs and numerous smaller rounded bodies outlining the fibrin network (Fig. 12.2c). These probably represented platelet and RBC debris. Free-floating intact RBCs were found, especially in the posterior vitreous space, indicating that the original clot structure had become less compact. Cellular invasion into the clot itself was sparse. When cells were present, their outlines were indistinct and they appeared as stellate cells with projections that merged indefinably with the general fibrillar structure of the clot (Fig. 12.2c). No aggregated or multinucleated cells were observed. On the surface of the clot, collagenous fibres formed an outer coat, within the layers of which there was abundant RBC debris (Fig. 12.2i).

Apart from the appearance of several large multinucleate giant cells, there was little change in the appearance of two week old clots. There was, instead, a general increase in most of the features just described, namely, large numbers of degenerating, free-RBCs within the posterior vitreous, substantial loosening of the clot framework suggesting clot lysis, thickening of the collagenous coat around the central clot, and a low-grade cellular response.

After three weeks, however, large quantities of amorphous granular debris that stained differently from intact or haemolysed RBCs with Mallory's phosphotungstic acid-haematoxylin stain (Fig. 12.2d) were observed. In addition, loose macrophage nuclei were noted, often in association with this material, on staining with haematoxylin and eosin (Fig. 12.2d, inset). These appearances suggested that aggregates of giant or multinucleate macrophages had lysed within the vitreous,

releasing their cell contents.

At one month, fresh mononuclear cells with very little cytoplasm (Fig. 12.2e) were seen in conjunction with older effete macrophages, which were in various stages of phagocytosis, aggregation and lysis, in clumps around still intact RBCs (Fig. 12.2f). The clot itself retained a well-defined reticular structure with some intact RBCs adherent to its framework, but most of the smaller debris had been removed from the clot (Fig. 12.21). The pseudocapsule which was very prominent on macroscopic examination was composed of condensed vitreous collagen with trapped RBCs forming a thick layer that enclosed the fibrin clot, and was continuous with collagenous material up to the ora serrata (Fig. 12.2j). No fibroblasts were seen at any stage.

From five weeks to two months after vitreous clot formation, RBCs, RBC debris, lysed cell products, fibrin content and the number of fresh macrophages decreased, whereas older macrophages in various stages of their natural life cycle persisted, and the collagenous pseudocapsule became more prominent and adherent to the posterior lens capsule. Iron staining within the macrophages was pronounced at this stage.

After two months, the histological appearances had altered considerably. Almost all of the original clot had been removed, leaving intact RBCs and aggregates of large multinucleate effete macrophages in varying proportions (Figs. 12.2g and inset, 3g and inset). The pseudocapsule remained, with RBCs and inflammatory cells within its layers, and it usually formed a membrane on the posterior lens surface or attached at various points to the ora serrata and the optic disc. No fresh invading cells were identified and all lysed cell material had been removed. Fibrin was not detectable after six weeks.

Between two and four months after clot formation, there was a reduction in the amount of residual material, but the response was variable. Several eyes became completely free of clot remnants, but in some, blood elements remained for many months. This usually took the form of intact, abnormally shaped RBCs (Fig. 12.2n), plus some very large, "spent" macrophages which were contained within a dense collagenous framework.

No other ocular pathology was noted in any eye throughout the duration of this study. In particular, the retinal architecture, seen by light microscopy, remained unaffected. No new vessel formation was observed on the retinal surface or the disc, and the retinal vessels themselves were normal. The first inflammatory cells that appeared in the vitreous after clot formation were noted in the area of the ciliary processes, in which the vessels were moderately dilated at this stage (two to three days). Iris, cornea and angle structures were unaffected by the presence of the vitreous clot. Some large inflammatory cells containing RBC debris and iron were occasionally seen within the stroma of the iris, but greater numbers of macrophages were noted within the trabecular meshwork and canal of Schlemm from the third to the twenty-eighth day. The time course varied from rabbit to rabbit, these figures representing the range of values. Vitreous clot material adhered firmly to the posterior lens capsule in all cases as already described, but the body of the lens was not affected.

DISCUSSION

Several aspects of the pathophysiology of vitreous haemorrhage resolution are unusual, which may be a reflection of the unique nature of this connective tissue. For instance, although the tissue thrombo-

plastic activity of the vitreous was found to be very low[382,421], whole blood clotted rapidly within gel vitreous (Fig. 12.1), in this case possibly as a result of Hageman factor activation by the vitreous (for a discussion of clotting mechanisms within the vitreous, see Chapter 4). In addition, platelet aggregation by vitreous collagen may be important in loculating the blood mass within the vitreous gel. It has previously been shown that when purified platelet preparations were injected into rabbit or owl monkey vitreous, instantaneous platelet aggregation occurred on contact with the collagen fibrils[106].

The fate of the various blood components after initial clot formation, and their effects on the structure of the vitreous have been reported on several occasions (see Chapter 10). Most of the RBCs that were released from the clot diffused into the posterior and inferior fluid vitreous where they were either phagocytosed by macrophages[224] or underwent haemolysis and caused the generalised opacification of the vitreous seen ophthalmoscopically. The RBCs appeared to be the seat of most of the phagocytic activity, but as has been shown biochemically in Chapter 8, their removal from vitreous was a slow process and was usually incomplete. The survival of RBCs within the vitreous for long periods of time has been recognised for many years[455], but the reason for their failure to excite an adequate cellular response remains unknown.

The cellular response to vitreous blood clots was also unusual in several respects. The early PMN cell reaction typical of most forms of inflammation was not seen[482,483] in this study. This confirmed the differential cell counts of Gray[194], who observed that mononuclear cells accounted for more than 90% of all leukocytes in the vitreous during the early stages of the cell response to vitreous blood deposits. This contrasted with other forms of vitreous inflammation,

such as those resulting from injection of bacteria and foreign particles, where PMN cells predominated at least in the early stages. In addition, the cellular response after vitreous haemorrhage in this study was sparse and was concentrated around isolated clumps of RBCs, while the main mass of the fibrin clot was poorly penetrated by cellular elements. However, an interesting cell type was observed within the fibrin clot, *ie.* a stellate or dendritiform cell (Fig. 12.2c and Fig. 12.21). Recent in vitro studies have shown that such cell forms occur when blood monocytes are induced to generate clotting of fibrinogen. Indeed, monocytes have been described as the "pro-clot" cell in contrast to basophils, which are known as "anti-clot" cells (see Chapter 2). It is possible that the appearances seen in vitreous clots represented the in vivo demonstration of such monocyte behaviour, although absolute identification of the cell type in the vitreous was not possible.

As to the origin of inflammatory cells in the vitreous, it is generally recognised that most macrophages present in inflammatory reactions are derived from precursors in the bone marrow[517]. The kinetics of this system have been extensively investigated for lesion of the peritoneum[525], spinal cord[265], skin[481] and brain[260]. The origin of vitreous inflammatory cells is not known, although Gloor[180] has suggested that the cells which appear within the vitreous after photocoagulation lesions of the retina are marrow-derived. It is possible that the macrophages that appear in the vitreous after haemorrhage are also marrow-derived, although the role, if any, of the tissue histiocyte, in this case the hyalocyte of Balazs[496] has not been fully investigated. In the present study, mononuclear cells first appeared in the region of the ciliary processes and rapidly increased in size as they ingested vast quantities of RBCs and detritus. Some of these cells later

migrated to the area of the trabecular meshwork[44], but the majority remained within the vitreous until by three weeks, having attained very large proportions, they underwent lysis. The appearance of fresh mononuclear cells within the vitreous at this stage suggested that a further chemotactic response had occurred. It is tempting to correlate this with the products of cell lysis which are known to be chemotactic in vitro[58]. After approximately six to eight weeks, however, no new cells were seen by light microscopy and large effete macrophages were found in association with unphagocytosed RBCs in residual clumps, many weeks and months after the original haemorrhage. Electron microscopic studies have shown that occasional young mononuclear cells were observed as long as eighteen months after haemorrhage (see Chapter 14).

The fate of the fibrin component has been investigated biochemically (see Part 2 of this thesis). The present histological study confirms the slow removal of fibrin from the vitreous (four to five weeks) and suggests that this delay may have been due not only to the low tissue fibrinolytic activity of the vitreous (see Chapter 7), but also to the absence of PMN leukocytes (see Chapter 5)[434]. In addition, the observation that platelet-fibrin aggregates within the vitreous initiate a poor macrophage response[106,160] was confirmed in this study.

The effects of haemorrhage on the gel structure of the vitreous are well recognised, especially detachment of the solid gel vitreous from the retinal surface[43,159,369]. However, the formation of a pseudocapsule by endogenous vitreous collagen has not been reported, although reference has been made to an "endothelial membrane"[217,369]. In addition, as the main clot mass was cleared away, the pseudocapsule remained as a dense collagen sheet with non-resorbed blood elements within its layers. It is noteworthy that in 45 consecutive blood

injections into the rabbit vitreous, no fibrosis or fibroblasts were seen. The controversy surrounding the concept that blood can stimulate fibroblastic activity has been discussed in Chapter 10, with the evidence for both aspects being considered. In the present study, despite the persistence of blood deposits in the vitreous for several months, no fibroblastic activity was detected. Collagenous material found in these cases appeared to be derived from a condensation of vitreous collagen forming a pseudocapsule around the clot that remained after most of the clot was reabsorbed.

CONCLUSION

The present study of the pathology of vitreous clot lysis has revealed a number of interesting features. After initial clot formation, the blood mass remained as a discrete structure for four to six weeks, after which it became considerably reduced in size. In the vitreous, several changes occurred, including liquefaction of the gel, posterior detachment of the solid vitreous, the appearance of prominent vitreous membranes, and the formation of a pseudocapsule around the clot. Small blood deposits remained in several eyes for many weeks.

The cell response to blood in the vitreous was slow to develop, was low grade, and was predominantly mononuclear from the onset of inflammation. Mononuclear cells developed into giant macrophages which formed large aggregates and multinucleate cells were frequently observed. The reasons for this atypical inflammatory cell response to blood in the vitreous are not known, but its effects may be to delay the reabsorption of blood from the vitreous.

The development of vitreous membranes and, in particular, the

pseudocapsule, appeared to result from coalescence of endogenous vitreous collagen fibrils, since fibroblasts were not observed in any tissue sections. It is suggested that fibroplasia is an unusual sequel to uncomplicated vitreous haemorrhage, and is associated with ocular diseases in which vitreous haemorrhage may be an incidental occurrence.

CHAPTER 13

ELECTRON MICROSCOPY OF VITREOUS CLOT LYSIS: HAEMOLYSIS AND VITREOUS MEMBRANE FORMATION

INTRODUCTION

In the previous chapter, analysis of the gross pathological and microscopic changes which occurred within the vitreous during clot resolution indicated that at least four morphological processes were taking place simultaneously, i.e. red cell destruction or haemolysis, fibrin removal or fibrinolysis, an inflammatory cell response, and the development of vitreous membranes, in particular a pseudocapsule around the clot. At least two of these changes were noteworthy; firstly, the inflammatory response was atypical in cell content and degree, and secondly, vitreous membranes developed in the absence of significant fibroblastic activity.

The lack of fundamental knowledge concerning the formation and structure of vitreous membranes has been alluded to by Freeman[155]. Several blood components such as platelets[106], leukocytes[278], lymphocytes[329] and haemoglobin[422] have been shown to produce vitreous membranes or strands, but such membranes were unlike those produced by whole blood (see Chapter 12) in that they were non-progressive, i.e. they remained unaltered for many months. There have been no ultrastructural studies of vitreous membrane formation during the process of vitreous clot resolution, although Smith et al[476] described the electron microscopic features of human vitreous membranes from specimens obtained at vitrectomy surgery. These membranes were composed of degenerating red cells, collagen fibres, and phagocytic cells of uncertain origin. In a separate ultrastructural study, Constable et al[106] described platelet-induced membranes in the owl monkey as strands of amorphous material in an unidentified matrix.

This chapter describes the ultrastructural appearance of vitreous membranes as they occurred during vitreous clot resolution in the rabbit.

In addition, the processes of intravitreal haemolysis and fibrin removal, which also have not been described previously at this level, are documented. In Chapter 14, a separate description of the cellular response to vitreous blood deposits is given, since several aspects of the cellular exudate were unusual and required separate analysis.

MATERIALS AND METHODS

ANIMALS

Seventeen mature New Zealand white rabbits were used in this study. The light microscope study reported in Chapter 12 indicated that the following time periods would provide useful material for examination: 5 minutes, one day, six days, three weeks, four weeks, six weeks, nine weeks, twenty weeks, and eighteen months after injection of blood into the vitreous. Accordingly, two animals were killed at each of these times, except for the five minutes stage, when one animal was studied.

INDUCTION OF VITREOUS CLOT

This has been described in Chapter 6. The enucleated eyes were fixed in 2-4% cacodylate-buffered glutaraldehyde at $4^{o}C$.

TISSUE DISSECTION and PREPARATION for electron microscopy has been described in Chapter 11.

ACID PHOSPHATASE ACTIVITY within selected tissue segments was demonstrated

by the method described in Chapter 11.

RESULTS

FIVE MINUTES

Clot formation occurred immediately after the blood was injected into the vitreous cavity. Clots of five minutes duration presented as discrete clumps which, on thick section, contained masses of normal packed red cells. The surrounding vitreous was clear and apparently unaffected by the presence of the blood. In particular, there was no evidence of red cell diffusion within the vitreous (Fig. 13.1).

TWENTY-FOUR HOURS

The surface of the clot consisted entirely of red cells which were less closely packed, but were connected to each other by thin strands (Fig. 13.2,13.3). Most of the cells had lost their biconcave appearance and were approximately spherical. Occasional smaller, rounded bodies, possibly platelets, were noted. Clefts and indentations on the clot surface were observed (Fig. 13.2), but the surrounding vitreous gel was normal. Transmission electron microscopy confirmed the alteration of red cell shape (Fig. 13.4) within the 24 hour old clots, and also demonstrated a considerable variation in electron density from cell to cell (Fig. 13.5). Some cells had surface blebs (Fig. 13.4), while others showed vacuolation of the cell cytoplasm (Fig. 13.6). In addition, the cytoplasm had a faintly inhomogenous granular appearance (Fig. 13.7). A few erythrocyte ghosts were seen, but in general red cell lysis was at an early stage. A thin continuous membrane surrounded

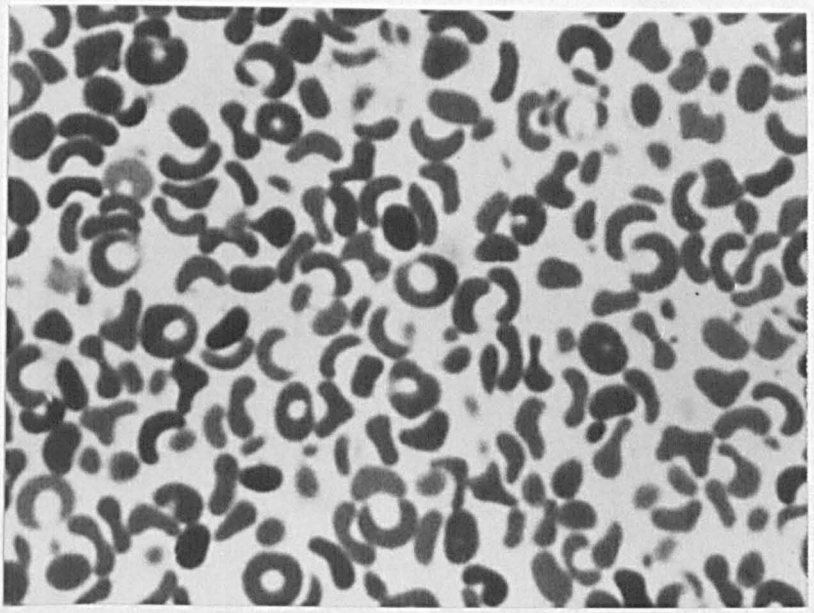

Figure 13.1 Thick section of vitreous clot five minutes after injection of blood into the rabbit vitreous. x 500

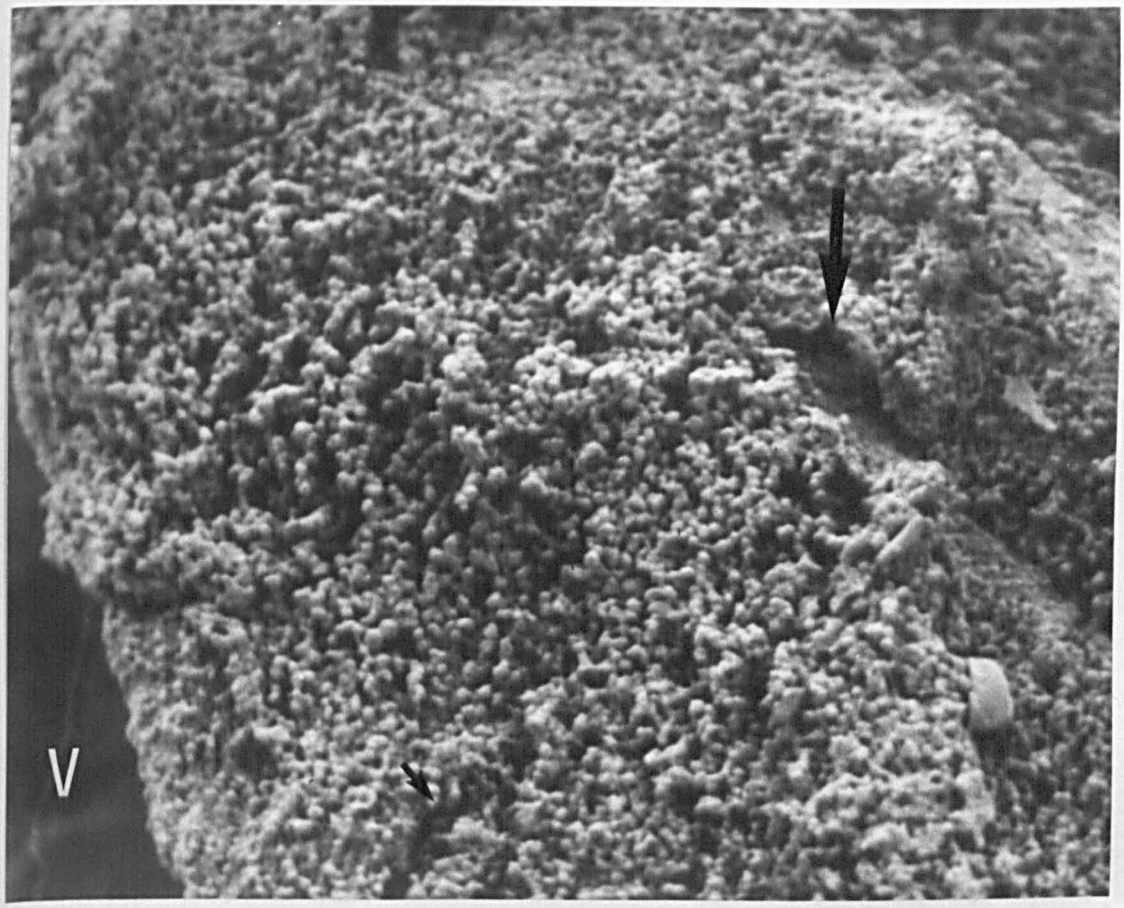

Figure 13.2 Twenty-four hour vitreous clot, S.E.M.*

Clot surface consists of masses of packed red cells. Surrounding vitreous gel (v) is normal. Clefts and indentations are seen on clot surface (arrows). x 200

*Footnote: S.E.M.; scanning electron microscopy.

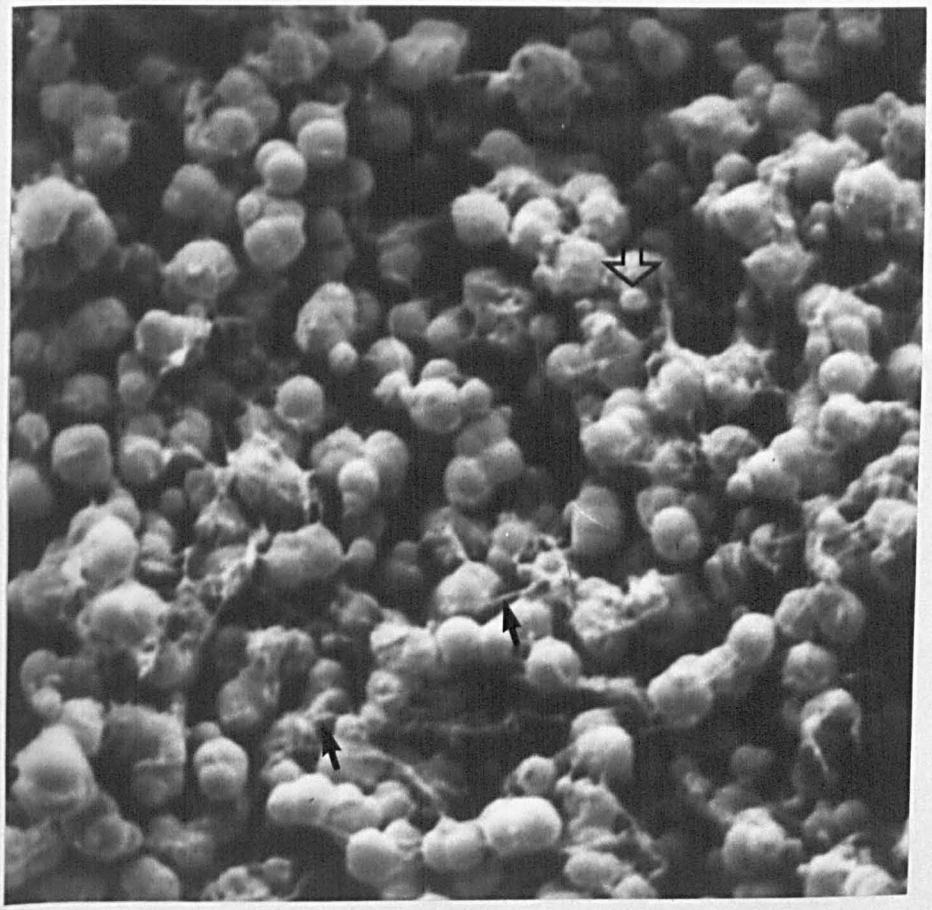

Figure 13.3 Higher power view of Fig. 13.2. Red cells are spherical and interconnect by fine strands (arrows). Smaller round bodies (arrowhead) also seen. x 500

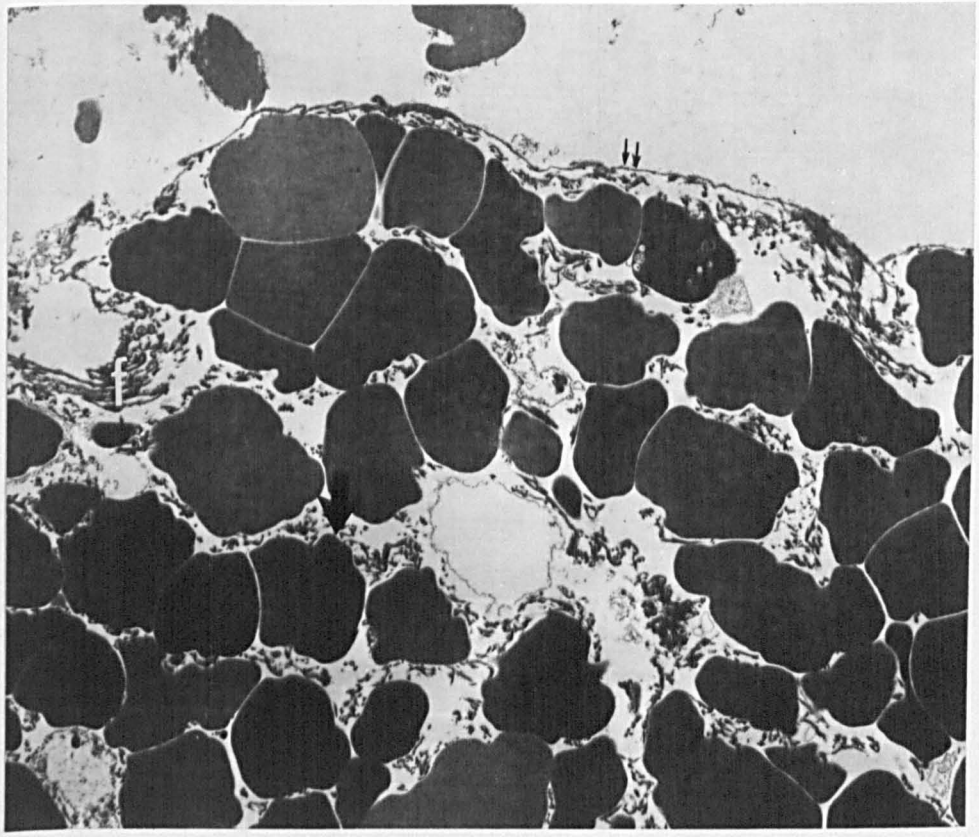

Figure 13.4 Twenty-four hour vitreous clot, T.E.M* Red cells within the clot show variable shape and some have surface blebs (arrow). A thin continuous membrane is seen on the surface of the clot (arrowheads). f, fibrin. x 3,500.

* Footnote: T.E.M.; transmission electron microscopy.

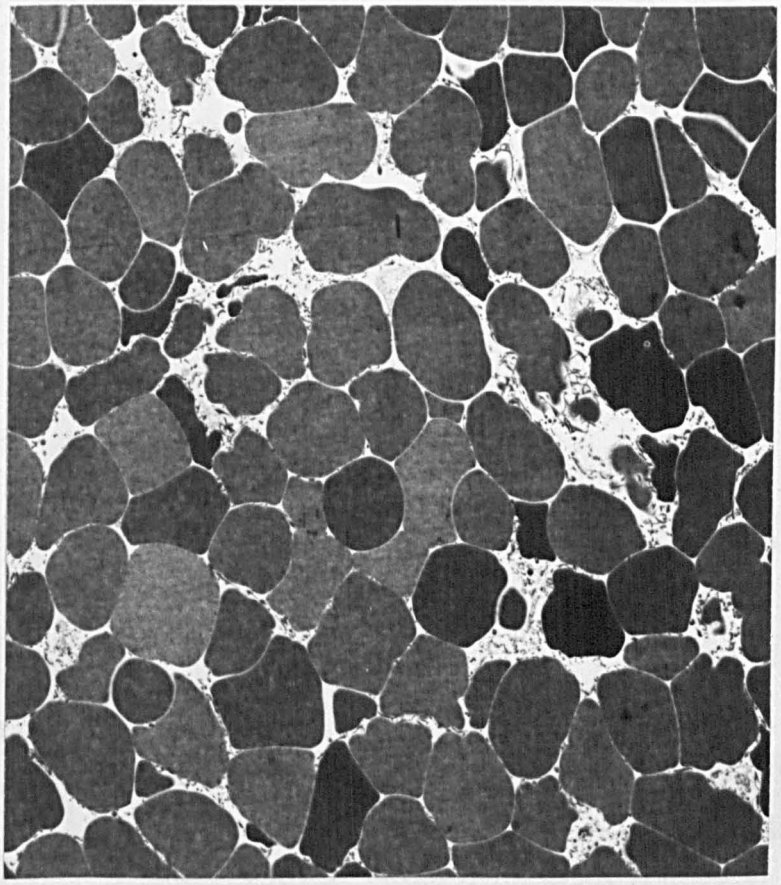

Figure 13.5 Twenty-four hour vitreous clot, T.E.M. Red cells within the clot show marked variation in electron density. x 2,800

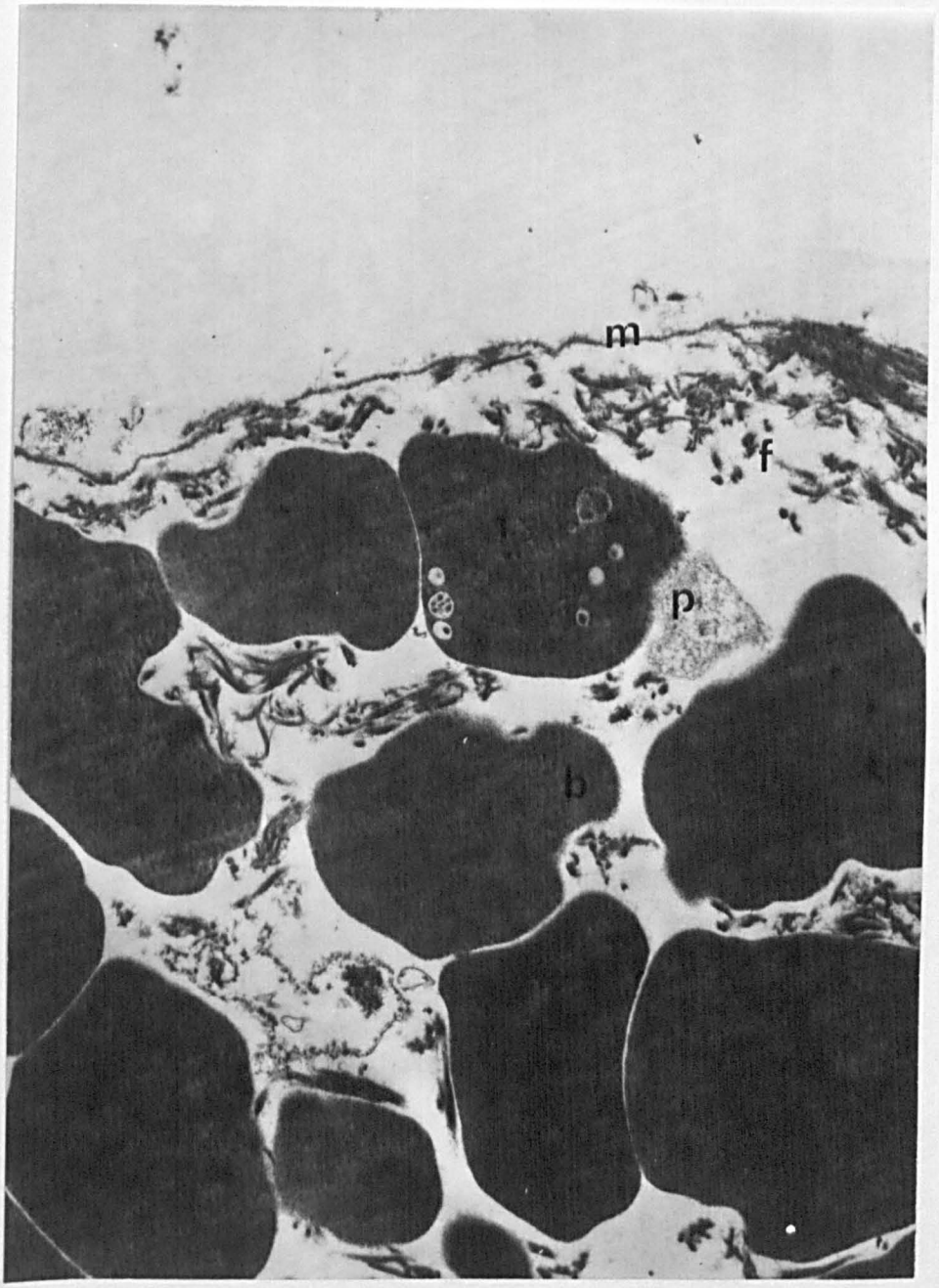

Figure 13.6 Twenty-four hour vitreous clot, T.E.M. A red cell in the centre of the figure shows several small round vacuoles containing electron dense bodies. p, platelet; f, fibrin; b, bleb; m, membrane. x 10,400

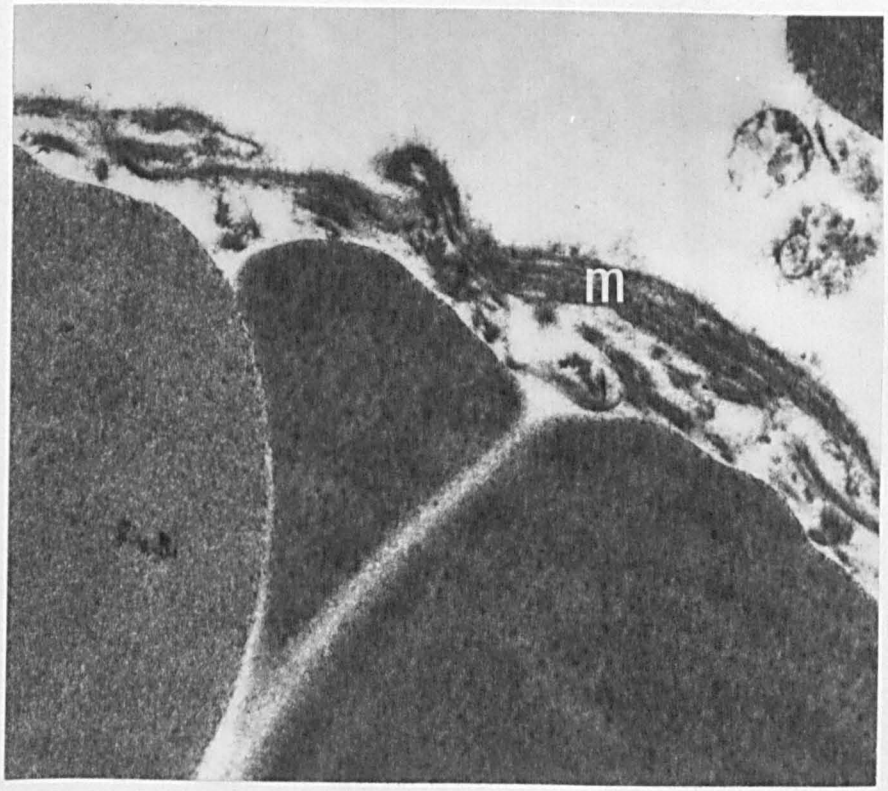

Figure 13.7 Twenty-four hour vitreous clot, T.E.M. Red cell cytoplasm has a faintly granular inhomogeneous appearance. Surface membrane (m) appears to be composed mainly of fibrin. x 21,000

the clot (Fig. 13.4) and considerable quantities of fibrin were seen on the inner surface of this membrane and also in the interstices of the clot. The nature of the surrounding membrane was less clear, but it appeared to be composed mainly of fibrin, intermingling with ill-defined fine vitreous collagen fibres (Figs. 13.6,13.7). Occasional lysed cell figures, possibly representing degranulated platelets or leukocytes, were present. No cellular invasion of the clot was seen.

SIX DAYS

Considerable liquefaction of the vitreous had occurred, and vitreous detachment was suggested by the presence of a distinct fibrous layer or "membrane" situated in the posterior vitreous between the retina and the clot (Fig. 13.8). Transmission electron microscopy of this layer revealed a double membranous structure, to which several electron dense round bodies were attached on its posterior surface (Fig. 13.9). Other electron lucent vacuolar structures resembling cytoplasmic organelles were seen. Numerous fine collagen fibrils were observed between this "membrane" and the clot surface, but none were seen on the retinal aspect of the membrane. Large quantities of electron dense amorphous debris intermingled with the fine fibrils in the region between the clot and the detached posterior vitreous face (Figs. 13.8, 13.9). This debris presumably resulted from changes occurring within the clot, and suggested that considerable red cell lysis had taken place. Scanning electron microscopy of the clot confirmed the marked loss of intact red cells, and also demonstrated the presence of a loose reticular membrane on the clot surface (Fig.13.10). Large cells, which were considered to be macrophages with extended processes and smooth contours, were seen on the surface of the clot, and were surrounded by numerous smaller rounded

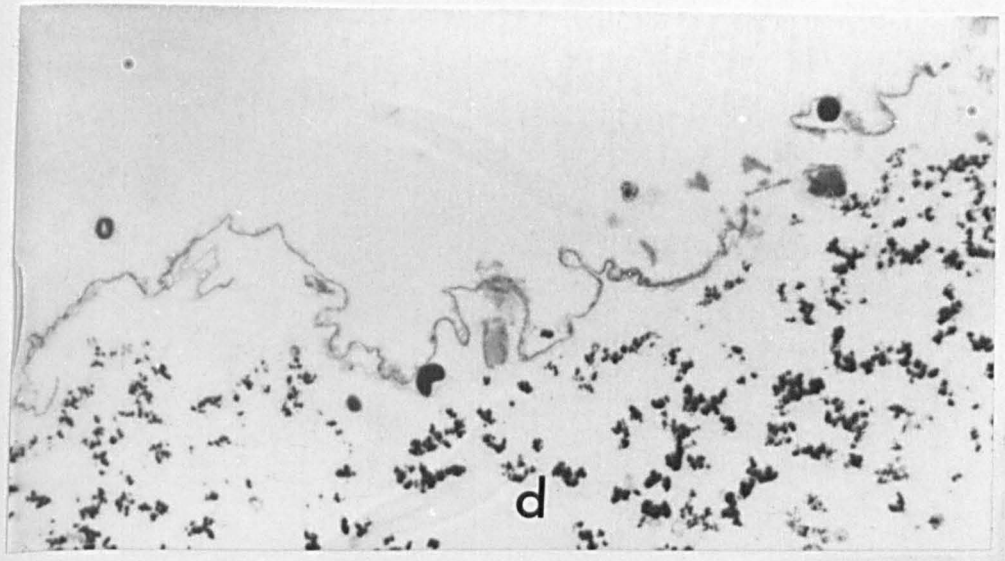

Figure 13.8 Six day old vitreous haemorrhage, thick section.

A distinct fibrous layer is noted posterior to the clot. Considerable clot debris (d) is seen between this layer and the clot, but none is shown on the retinal aspect of this layer (upper field). x 340

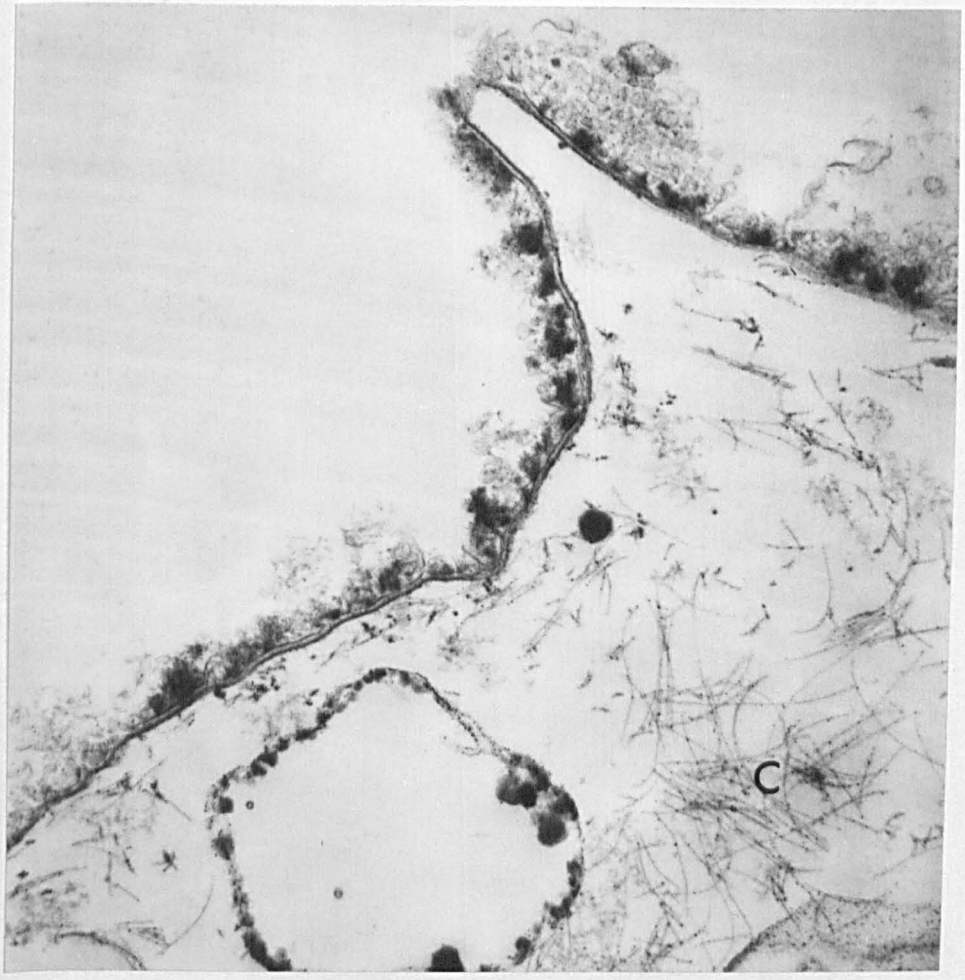

Figure 13.9 Six day old vitreous haemorrhage, T.E.M. Ultrastructure of fibrous layer posterior to clot. A double membranous structure is shown, with numerous electron dense particles and electron lucent vesicles on its retinal aspect (upper and extreme right fields). C, fine collagen fibrils. x 13,000

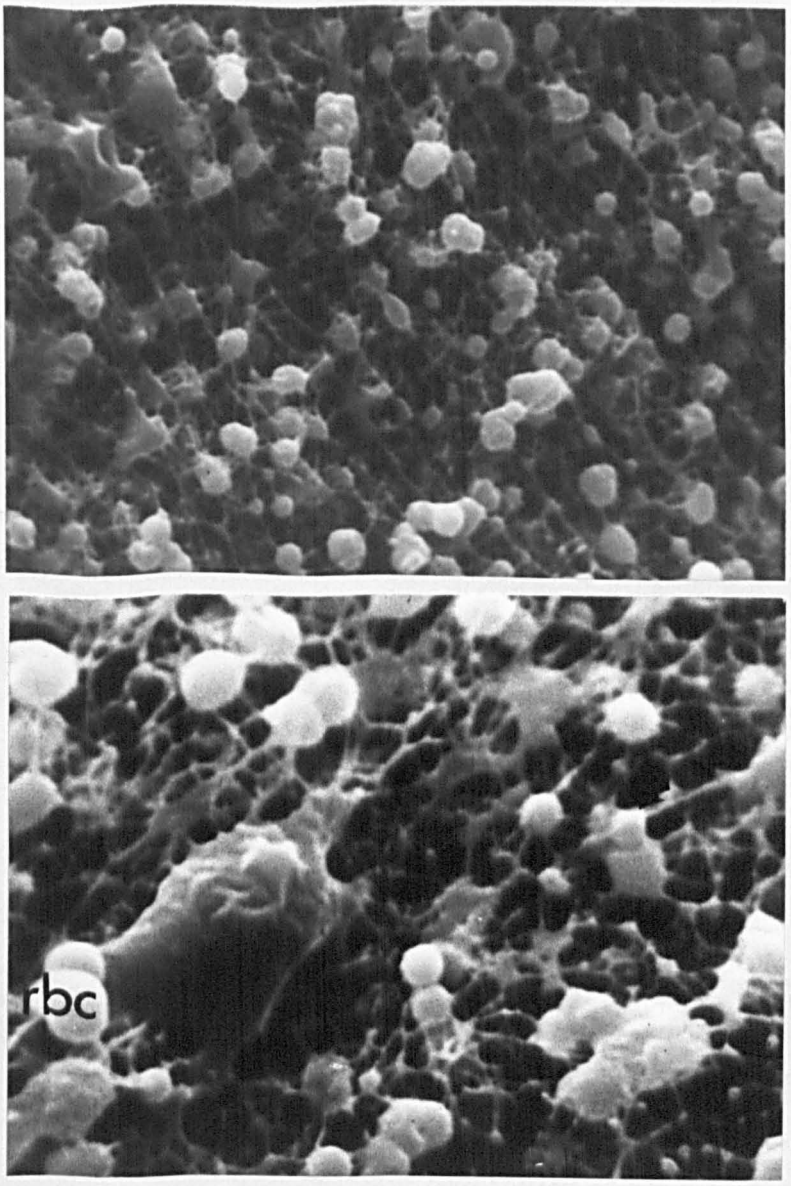

Figure 13.10 Six day old vitreous clot, S.E.M. Clot surface has acquired a loose reticular appearance. Note marked variation in size of residual red cell bodies. Few intact red cells are shown. x 1,000.

Figure 13.11 Six day old vitreous clot, S.E.M. A smooth-surfaced spread macrophage is shown on the surface of the clot. RBC, red cell. Arrow, unidentified round body, probably residual red cell body. x 2,000.

bodies whose identity was unclear (Fig. 13.11). At higher magnification, the matrix of the surface membrane was seen to be composed of a complicated network of interconnecting strands (Fig. 13.11) with particles of amorphous debris bridging the inter-strand spaces. Considerable changes were also observed in the red cells which remained in the centre of the clot. Closely packed cell figures were still identifiable, but their markedly inhomogeneous and granular cytoplasm indicated that extensive haemolysis had occurred (Fig. 13.12). In some areas, red cell membranes were easily detected (Fig. 13.13), whereas in others cell membrane disruption was clearly evident (Fig. 13.13). Intact cells and partially haemolysed residual cell bodies were also noted within the clot (Fig. 13.12). However, intact cells were more commonly found in the posterior vitreous cavity some distance from the original clot. By this stage, a mononuclear cell response was established (see Chapter 12), but transmission electron microscopy showed no evidence of invasion of the clot substance. Inflammatory cells were noted only on the surface of the clot (Fig. 13.11). Acid phosphatase activity was prominent within the macrophages, but none was present on the red cell membranes.

THREE TO SIX WEEKS

During this period, vitreous detachment proceeded to completion. The clot became enclosed in a prominent sheet of tissue, mainly derived from the posterior hyaloid surface. This layer has previously been described as a pseudocapsule (see Chapter 12). Scanning electron microscopy of the pseudocapsule showed that it varied in density, over different areas of the clot, from a well formed, thick structure

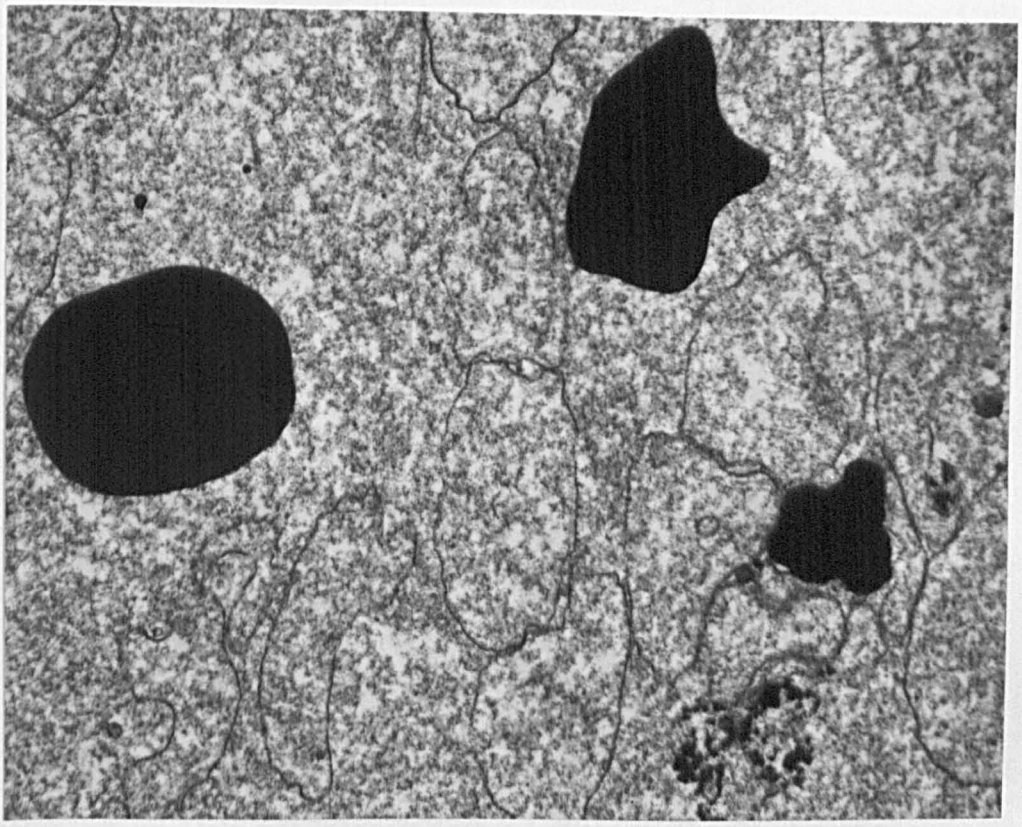

Figure 13.12 Six day old vitreous clot, T.E.M. View shown is from centre of the clot. Lysed red cell figures are present, many with intact cell membranes. Intact red cells and residual red cell body also seen. x 4,100.

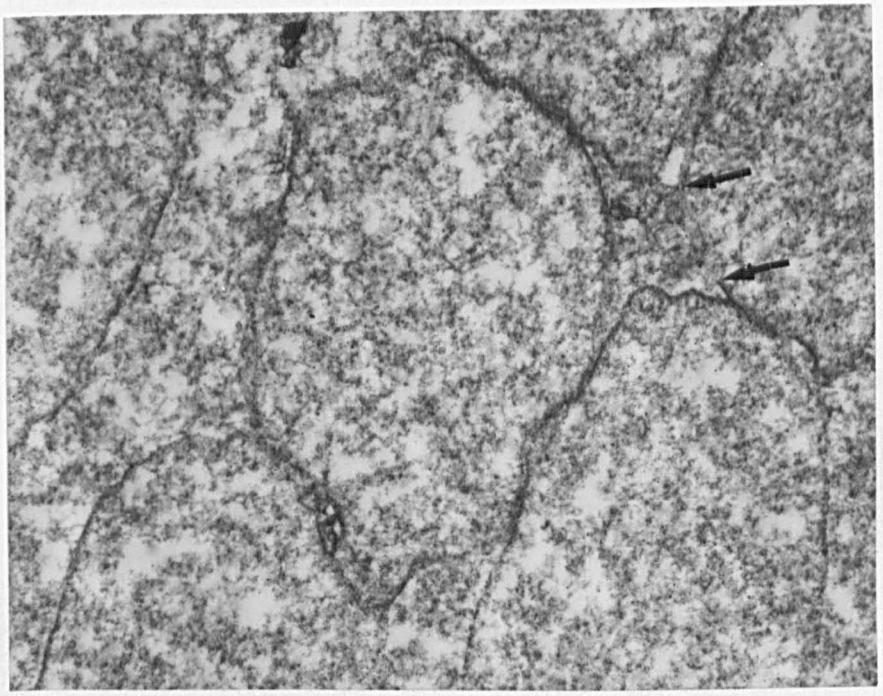

Figure 13.13 Six day old vitreous clot, T.E.M. Lysis of red cell membranes is shown (arrows). x 16,000

to a membrane with a loose reticular framework (Fig. 13.14). By transmission electron microscopy the pseudocapsule was seen to comprise parallel bundles of thin fibrils, presumably vitreous collagen, which enclosed red cell debris and other amorphous material (Fig. 13.15). No fibroblasts were seen associated with the pseudocapsule, but cellular activity was observed in the form of rounded cells, with the appearance of macrophages, which were adherent to the surface of the vitreous membranes and pseudocapsule (Fig. 13.16). Numerous residual red cell bodies, and a few intact red cells, were also adherent to the markedly irregular surface of the pseudocapsule (Fig. 13.14). The attachment of this material to the pseudocapsule may have occurred during the rapid freezing process used for scanning electron microscopy, particularly since most of the lysed red cells now occupied a position in the posterior vitreous cavity behind the detached vitreous face (Fig. 13.17). In addition to ghost cells and electron dense haemolysed particles (Fig. 13.18), intact red cells in the posterior vitreous showed various signs of degeneration such as intracellular vacuolation and sickling (Fig. 13.17). Acid phosphatase activity was prominent on these cells, and also within the vacuoles (Fig. 13.19). Between four and five weeks, the clot was still recognisable as a pale mass enclosed by the pseudocapsule (see Chapter 12). A surface view of the clot was obtained by dissecting the pseudocapsule from the clot while the globe was in the fixation fluid. At this stage of resolution, cellular elements were absent from the clot, which presented instead as a loose honeycombed structure (Fig. 13.20). The alveolar spaces or lacunae of the honeycomb were empty, and the walls of each lacuna were formed by a thin layer of amorphous debris. Interalveolar communication was complete by a complex system of small tunnels. Previous

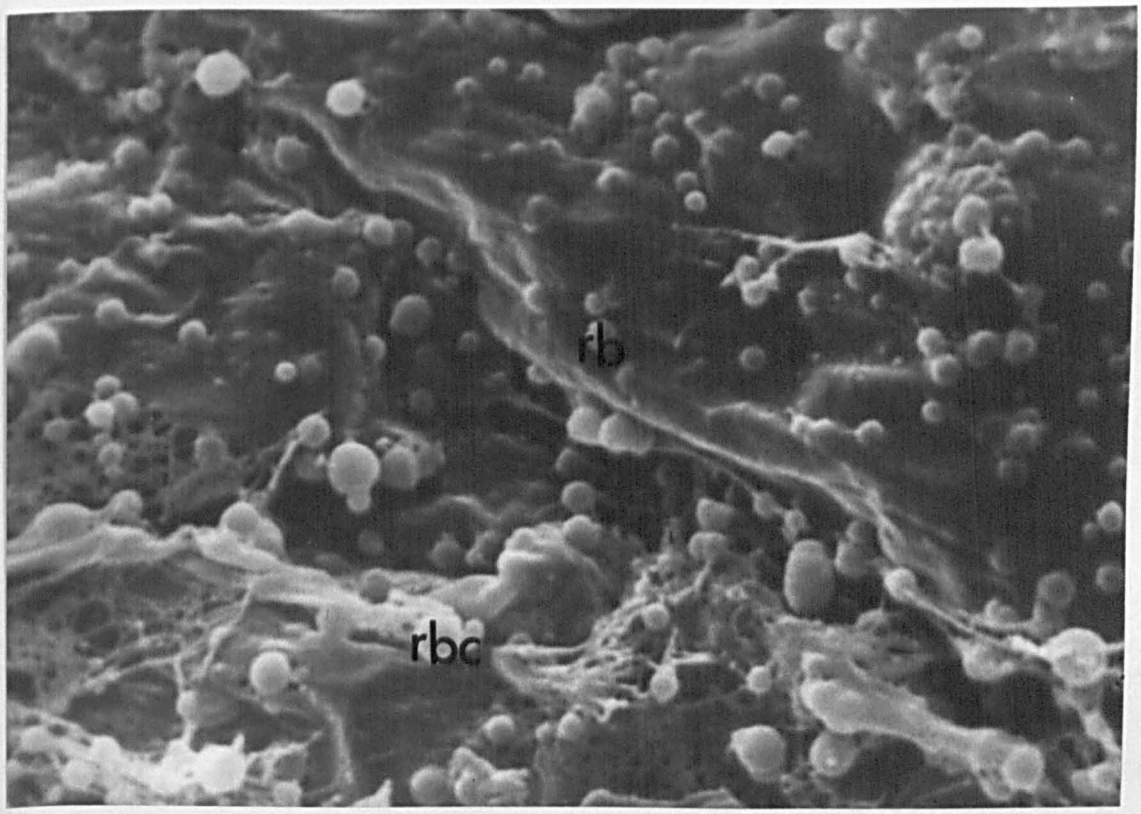

Figure 13.14 Three week vitreous haemorrhage, S.E.M. Surface view of pseudocapsule around clot, shown here as a dense irregular sheet of tissue. rb, residual red cell body; rbc, intact red cell. x 2,000

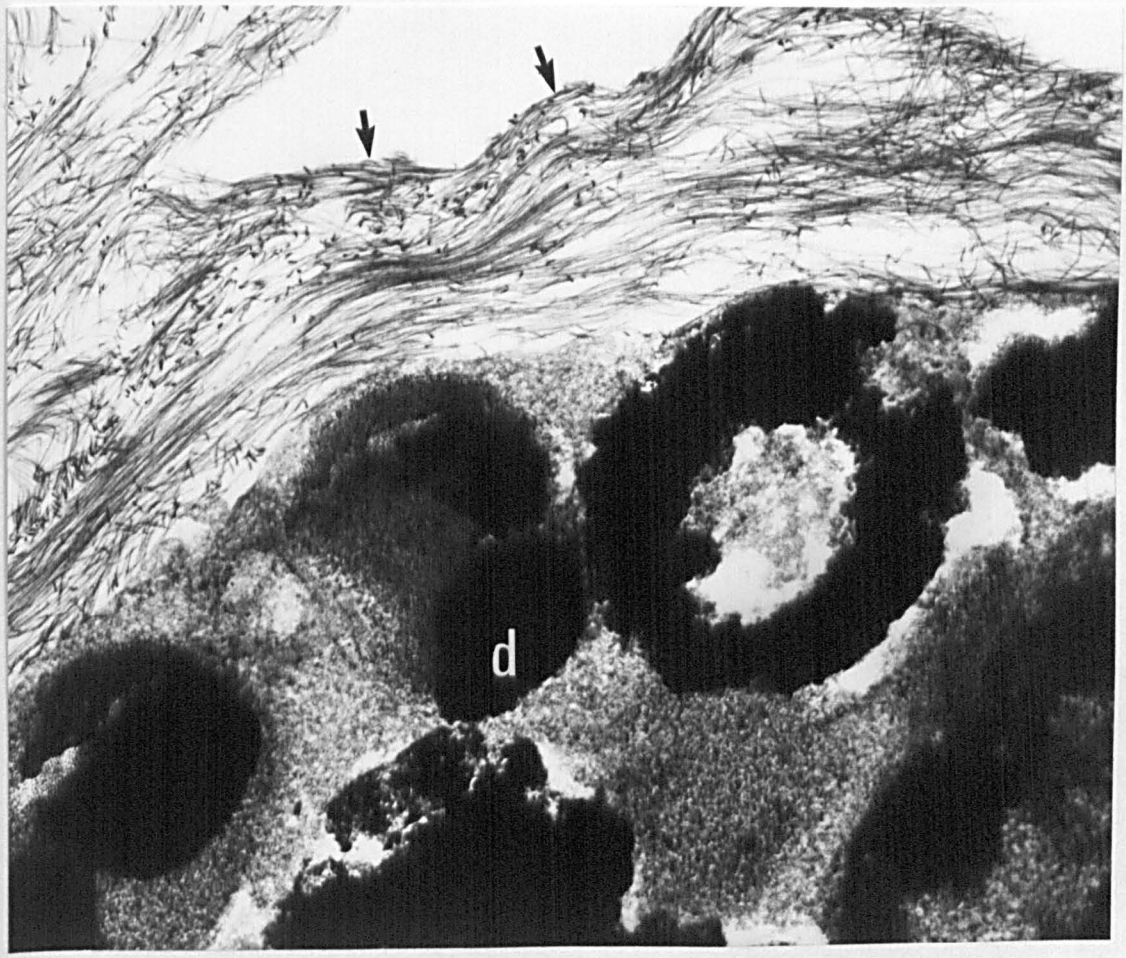

Figure 13.15 Three week vitreous haemorrhage, T.E.M. Section taken through pseudocapsule. Note parallel array of thin fibrils. Arrows outline surface of pseudocapsule. d, red cell debris within macrophage cytoplasm. x 26,800

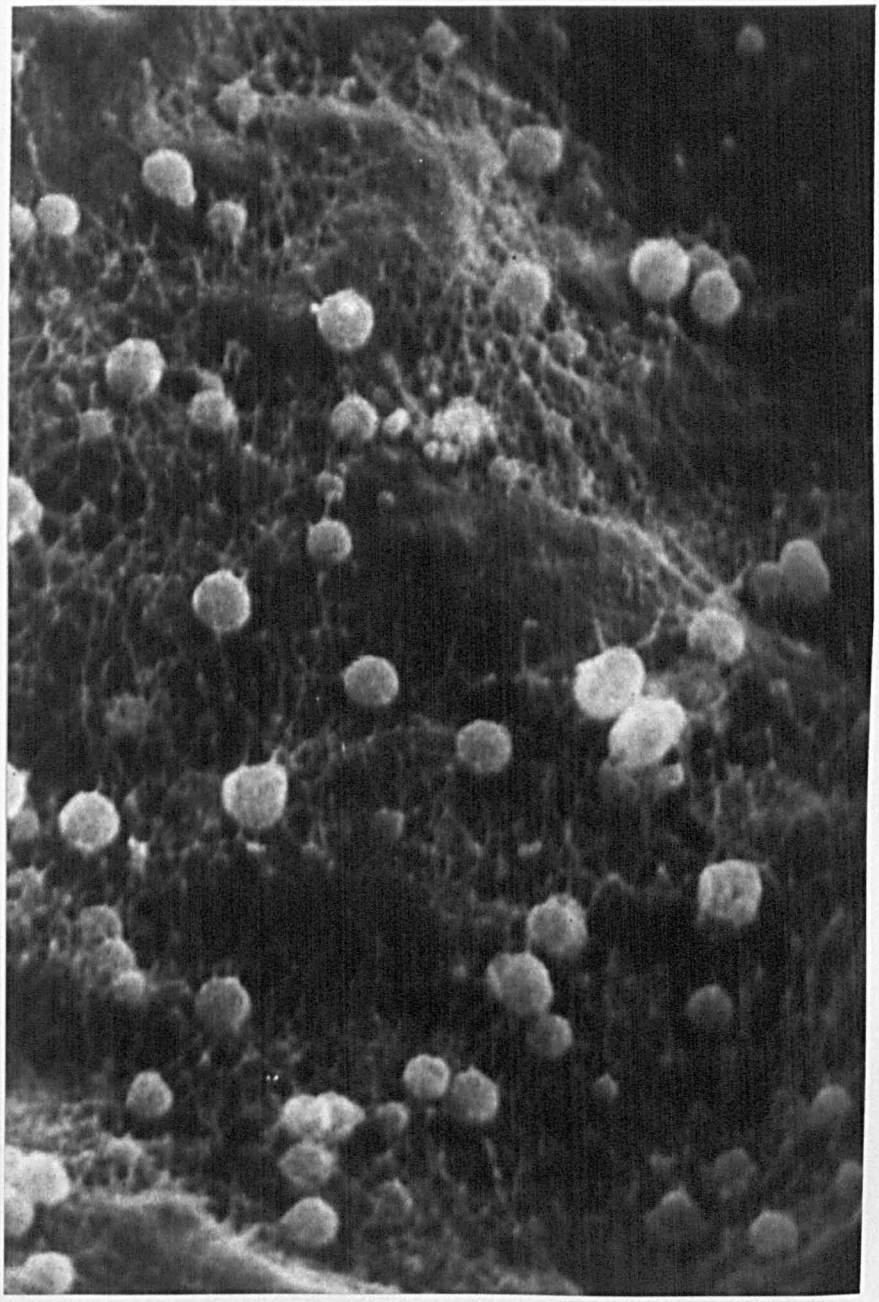

Figure 13.16 Four week vitreous clot, S.E.M. Rounded macrophages on surface of pseudocapsule around clot. x 2,000

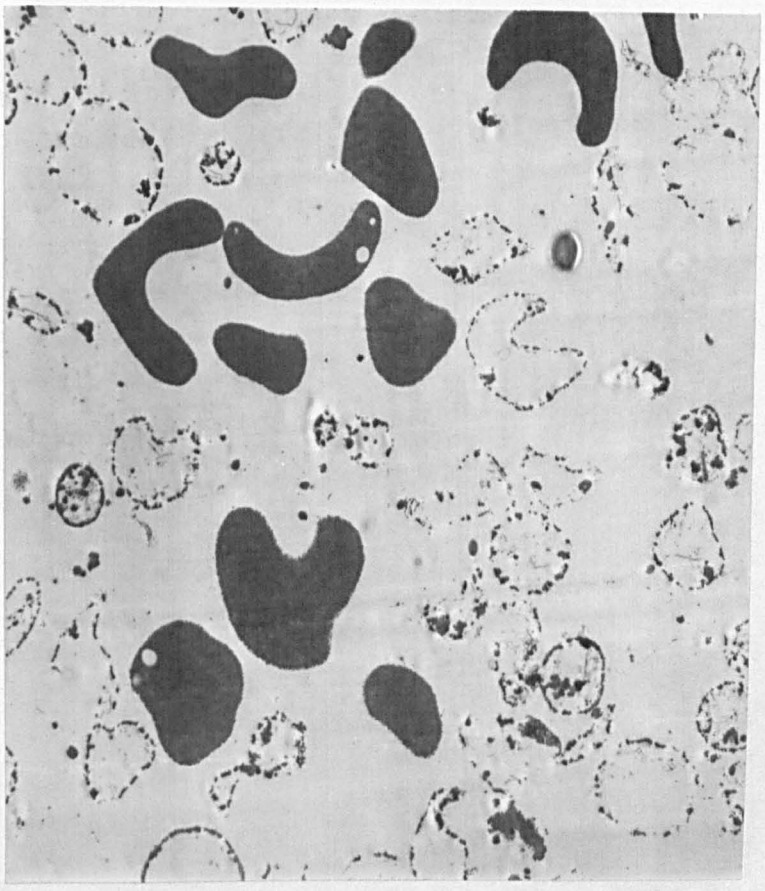

Figure 13.17 Four week vitreous haemorrhage, T.E.M. View of posterior vitreous cavity, behind the clot. Note numerous red cell ghosts and sickling and vacuolation of intact red cells. x 3,400

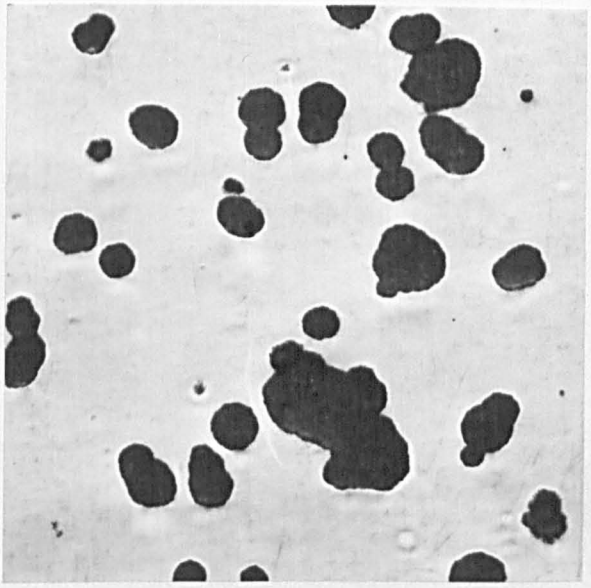

Figure 13.18 Four week vitreous haemorrhage, T.E.M. View of posterior vitreous cavity, behind the clot. Note large numbers of residual red cell bodies and electron dense haemolysed particles.

x 3,300

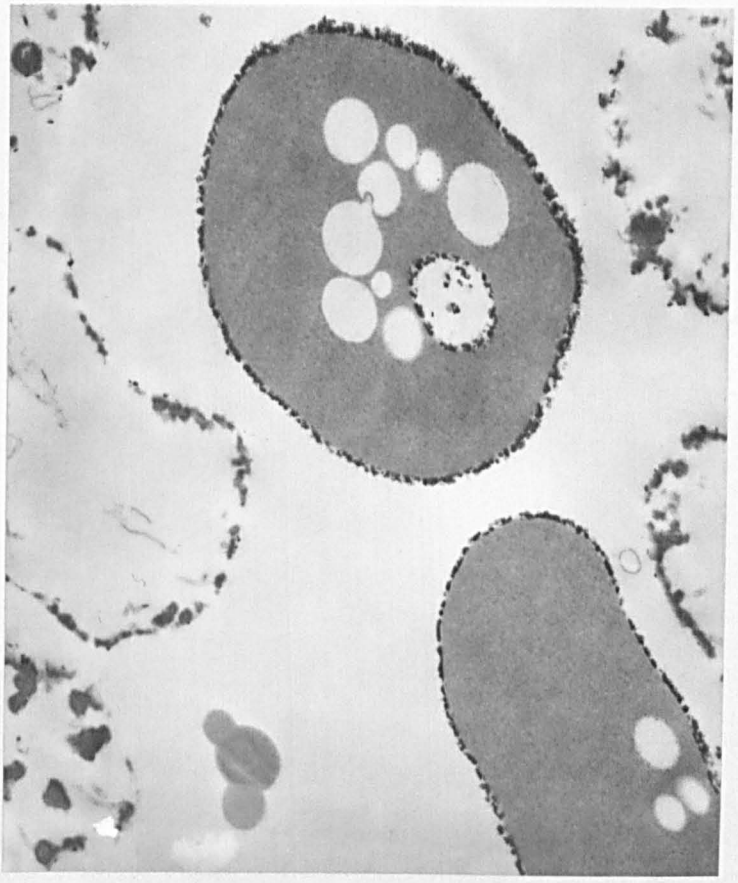

Figure 13.19 Three week vitreous haemorrhage, T.E.M. Note strong acid phosphatase reaction product on red cell surface and within a vacuole. Ghost cells are negative for acid phosphatase. x 13,300

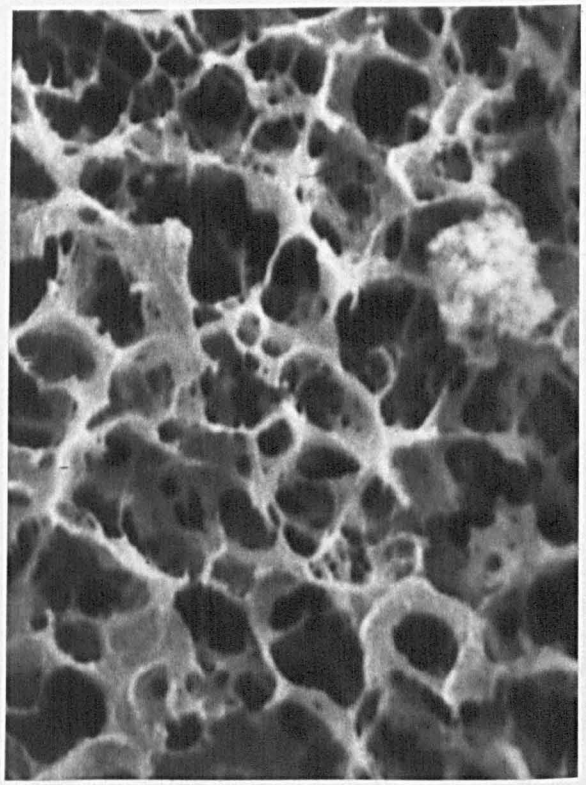

Figure 13.20 Four week vitreous clot, S.E.M. The pseudocapsule has been removed from the clot to obtain a surface view of the clot itself. Note the absence of cellular elements and the unusual honeycombed appearance. The lacunae of the honeycomb are empty and interalveolar communication exists via short tunnels. x 1,000

studies have shown that the framework of this structure consisted of fibrin (see Chapter 12). By the sixth week, the clot was no longer recognisable as an intact structure and it was presumed that clot lysis had finally occurred. There was, however, still considerable clot debris within the vitreous, mostly as loose vitreous "membranes", the most prominent of which was the pseudocapsule.

NINE TO TWENTY WEEKS

During this period, there was a progressive reduction in the amount of haematogenous material in the vitreous. Generally it took the form of vitreous "membranes" which varied greatly in size and composition. The pseudocapsule remained as a dense sheet of tissue (Figs. 13.21, 13.22) which enveloped a mass of red cell debris. Transmission electron microscopy (Fig. 13.22) showed that the pseudocapsule was composed of interwoven bundles of thin fibrils, presumably vitreous collagen. Fibroblasts were not observed in the pseudocapsule. Other vitreous membranes were less dense than the pseudocapsule and two types of membrane were identified, namely cellular and acellular. Cellular membranes (Fig. 13.23) occurred as aggregates of macrophages enclosed within a thin layer of collagen-like material. Such aggregates often assumed very large proportions and contained essentially pure populations of giant macrophages. Numerous phagocytic inclusions were observed by transmission electron microscopy within the cytoplasm of these cells (Fig. 13.24). In addition, the shape and size of the phagosomes was extremely variable and included recently ingested intact red cells, lipid droplets, myelin whorls, electron lucent vacuoles and electron dense bodies (Fig. 13.24). In contrast, acellular membranes were formed by the coalescence of vitreous collagen fibrils, which

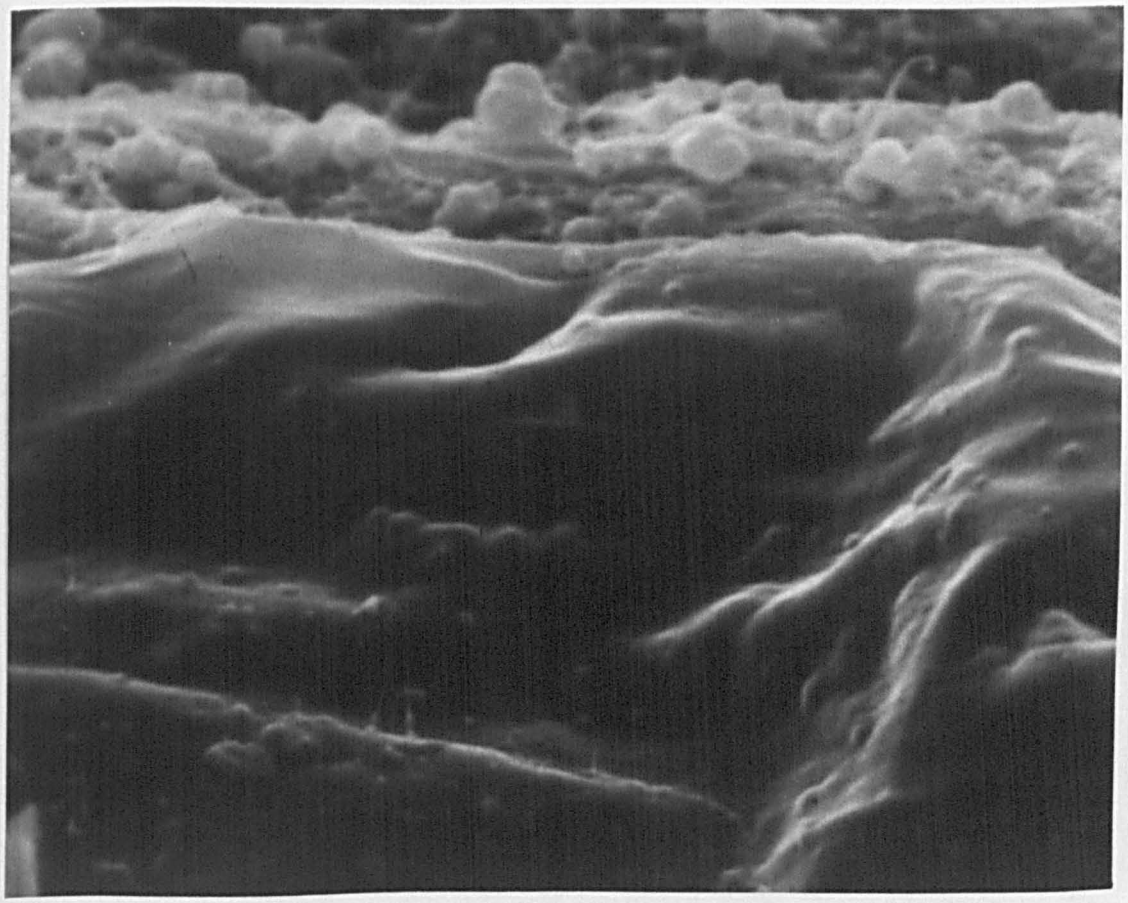

Figure 13.21 Ten week vitreous haemorrhage, S.E.M. Surface view of pseudocapsule. Note residual clot debris in upper half of field. x 2,000

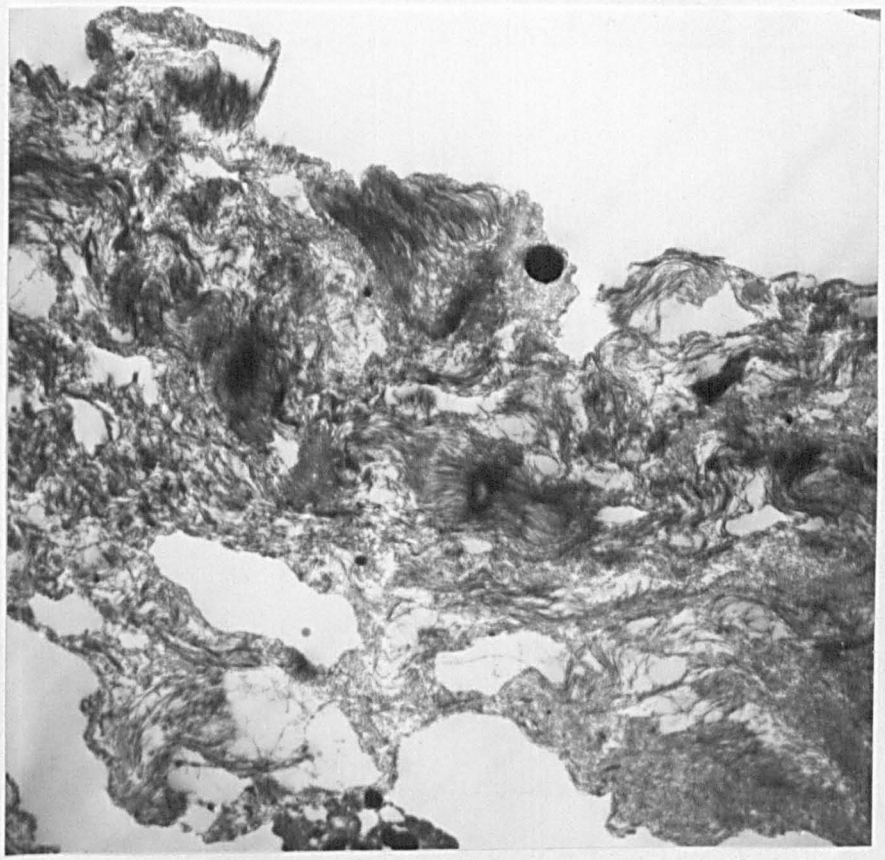

Figure 13.22 Ten week vitreous haemorrhage, T.E.M. Section through pseudocapsule. It is composed of interwoven bundles of thin fibrils, most likely derived from coalescence of vitreous collagen fibrils. x 3,800

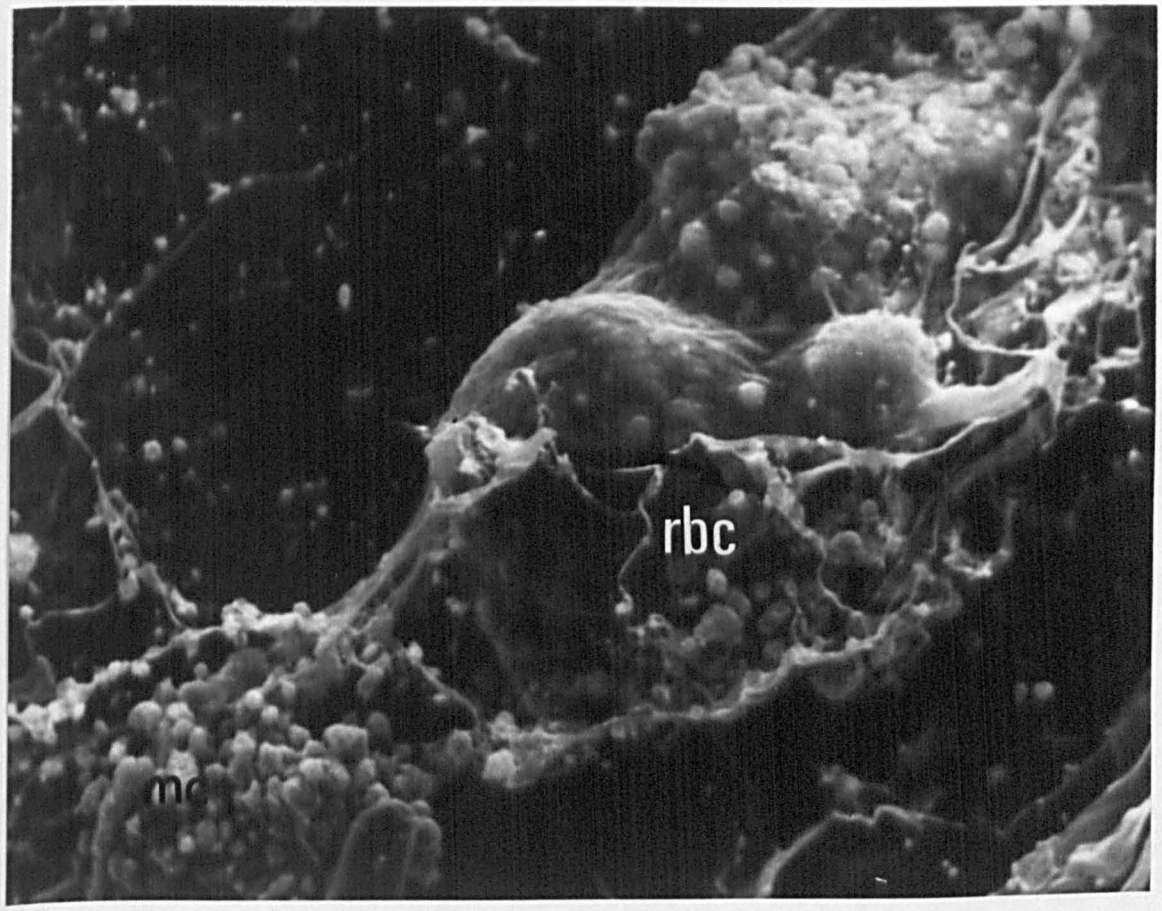

Figure 13.23 Twelve week vitreous haemorrhage, S.E.M. Cellular vitreous membrane composed of a mass of aggregated macrophages (mc) enclosed by a thin sheet of tissue, presumably collagen. rbc, intact red cell. x 180

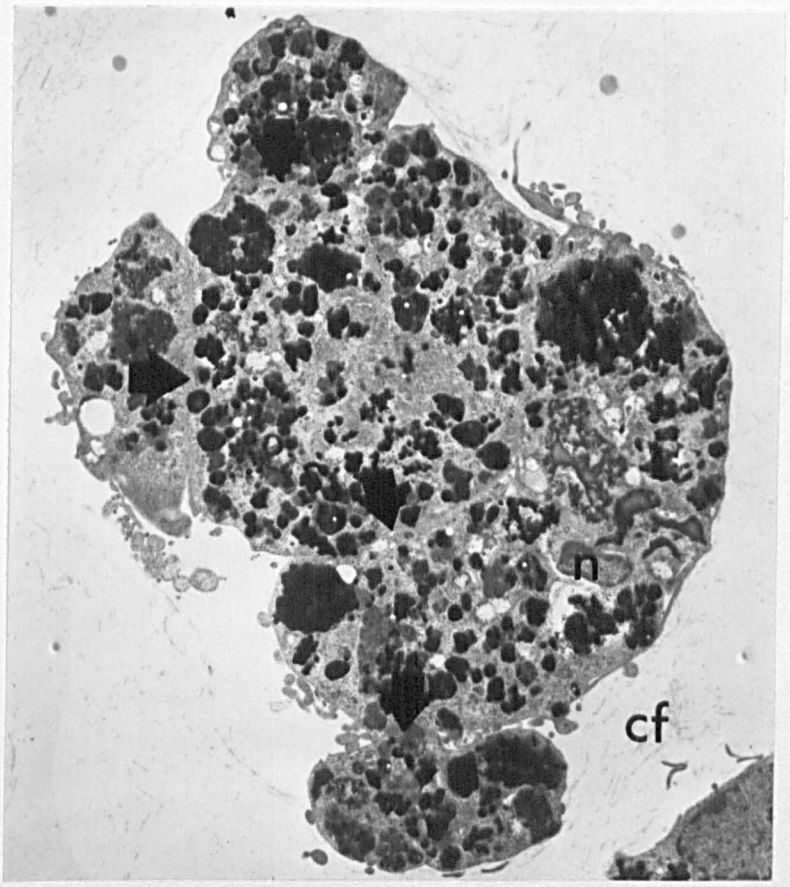

Figure 13.24 Twenty-week vitreous haemorrhage, T.E.M. Aggregate of five macrophages within a cellular membrane.

n, nucleus; cf, collagen fibrils; arrows, outline of cell limits.

x 8,400

occurred during the process of vitreous detachment. Acellular membranes occurred more frequently in areas remote from the original clot and presented as cords of fibres, frequently spanning the distance between the posterior lens capsule and the vitreous base or the peripheral retina (Fig. 13.25). Later, they retracted into a retrolental position, in close apposition to the more cellular membranes which were associated with the amorphous remains of the clot. The composition of the acellular membranes consisted of dense bundles of poorly banded parallel thin fibrils coursing in a three-directional network (Fig. 13.26). No fibroblasts were seen in association with either the cellular or acellular membranes. Complete detachment of the vitreous cortex was confirmed by surface scanning electron microscopy of the retina (Fig. 13.27). Loose strands of cortical vitreous fibrils were seen in some areas of the retina, but most of the surface was free of fibrillar material. Occasional intact red cells and large macrophages were still observed up to twenty weeks after vitreous clot formation (Fig. 13.27).

EIGHTEEN MONTHS

A residual pseudocapsule could not be identified. Only very fine vitreous membranes were observed and these were both cellular and acellular in type, although the latter predominated. Their ultrastructural morphology differed only in degree from the appearances of membranes which occurred between nine and twenty weeks. In general, there was a gradual reduction in the cellularity of membranes with time.

Figure 13.25 Twenty week vitreous haemorrhage, S.E.M. Acellular membrane, composed of thick cord-like fibrils and closely apposed to posterior lens surface (L). x 75

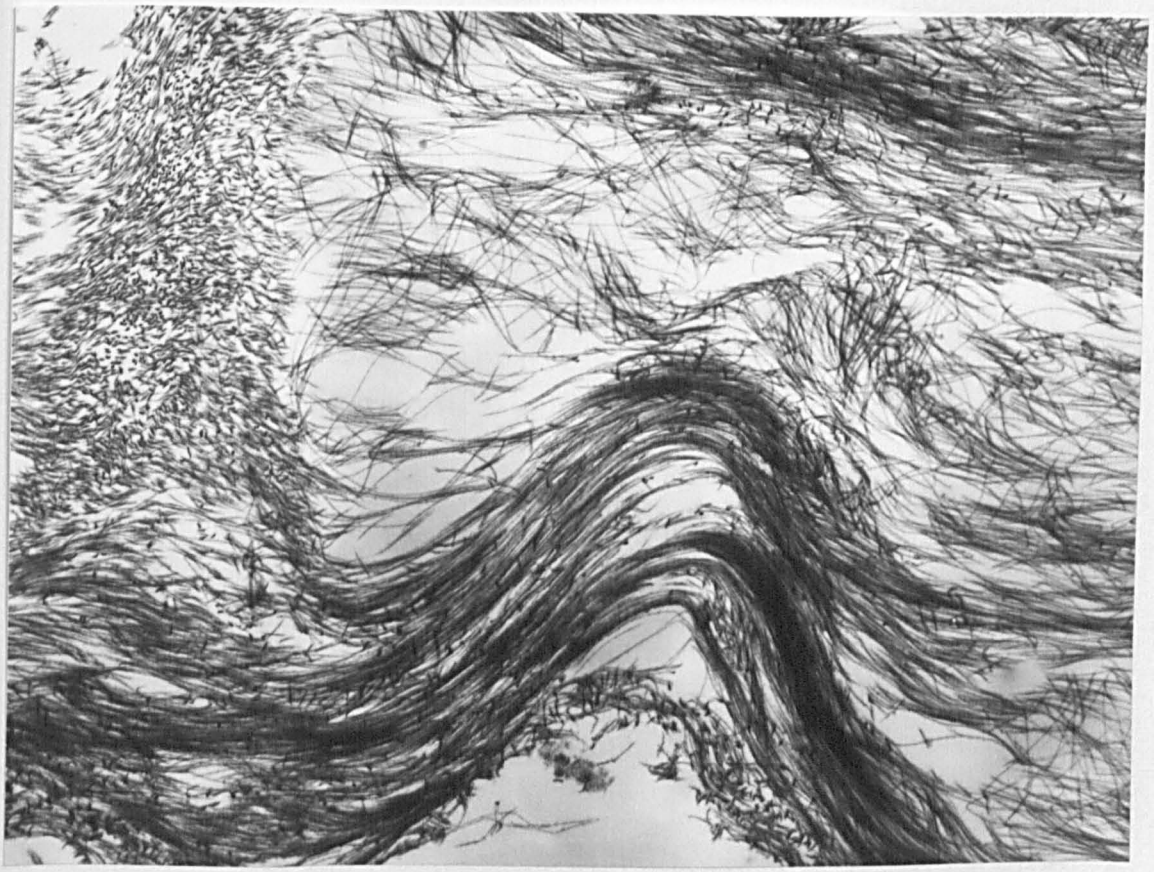

Figure 13.26 Twenty week vitreous haemorrhage, T.E.M. Acellular membrane composed of thin collagen fibrils, without clear banding pattern. Note absence of fibroblasts. x 20,200

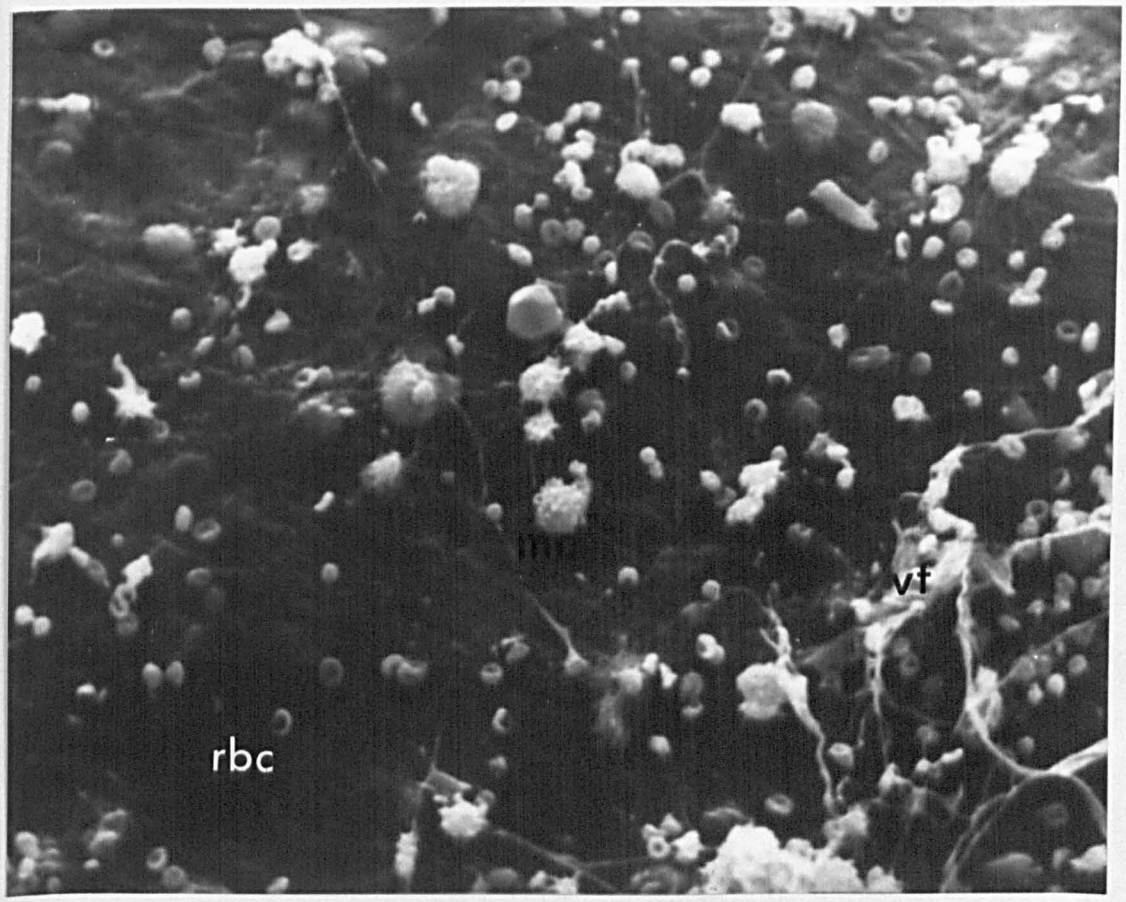

Figure 13.27 Twenty-week vitreous haemorrhage, S.E.M. View of retinal surface showing detached vitreous fibrils (vf), intact red cells (rbc) and macrophages (mc). x 250

DISCUSSION

During the reabsorption of blood from the vitreous, several repair processes are known to occur simultaneously, including haemolysis[196], fibrinolysis (see Chapter 8), and the initiation of an inflammatory response[194]. Previous experimental studies have emphasised the importance of haemolysis in this process[196,223,424], but although Regnault[424] has described the intravitreal degradation of haemoglobin, there have been no reports which specifically follow the course of red cell lysis at an ultrastructural level. In the present study, evidence of haemolysis was detected as early as twenty-four hours after the intravitreal injection of blood, by changes in red cell shape and size, by vacuolation and inhomogeneity of the cell cytoplasm, and by variation in electron density of the red cells. Progressive haemolysis continued for some time and led to the removal of most of the red cells from the vitreous by ten weeks, but significant numbers of intact red cells were present after several months (Fig. 13.27). The prolonged survival of red cells within the vitreous has already been noted (see Chapter 12).

The mechanism whereby red cells undergo lysis within the vitreous is not clear. Experimental studies have shown that in other tissues and organs such as the spleen[470], bone marrow[500], bronchial tree[103,104] and subarachnoid space[306], red cell lysis is an intracellular process, subsequent to the engulfment of whole red cells by mononuclear phagocytes. The present study has shown that this process also occurred in the vitreous, and the pattern of cell lysis was similar to that described by Edwards and Simon[131]. Digestion of engulfed red cells was often incomplete, however, with the result that large numbers of residual red cell bodies were observed in mature macrophages (Fig. 13.24). These

macrophages eventually underwent lysis within the vitreous (see Chapter 12), thereby releasing numerous partially digested red cell fragments into the extracellular space.

In addition to intracellular red cell lysis by macrophage phagocytosis, considerable extracellular lysis (autolysis) of red cells was noted. A similar haemolytic process has been reported for blood deposits in rat peritoneum[135] and Papadimitrou et al[385] have described the progressive extracellular degradation of red cells induced by phagocytes. Although it is generally accepted that extracellular lysis is a less common means of disposing of red cell material[470] in the vitreous, it appeared to constitute the major pathway for red cell removal. Lysis of red cells began before the appearance of inflammatory cells, and may have been due to the unfavourable environment provided by the vitreous. Although it is unlikely that differences in the osmolality[124], pH[117], or ionic composition[419] between plasma and vitreous had an effect on red cell survival, it is possible that the significantly lower oxygen tension[235] and reduced glucose concentration[419] within the vitreous initiated autolysis of red cells[466]. The ability of the red cells to withstand these conditions probably varied with the age of the cell, and would therefore have been reflected by different cellular rates of lysis, as is suggested by their varying electron density at 24 hours (Fig. 13.5). This concept was further supported by the sickling of red cells within the vitreous (Fig. 13.17).

Extracellular lysis of red cells continued after the appearance of inflammatory cells in the vitreous, and by this stage macrophage exocytosis may have contributed to the haemolytic process, particularly since lysis was prominent on the surface of the clot after six days, where most of the macrophage activity was observed. Macrophages are known to secrete lysosomal enzymes selectively in response to inflammatory stimuli[116,550]

and lysosomes contain specific haemolytic enzymes[118]. In the present study, it was interesting to note that acid phosphatase activity was not detectable on red cell membranes until three weeks after vitreous clot formation, by which time similar activity was particularly high within macrophage lysosomes. Although some of the activity on the red cells probably represented endogenous red cell acid phosphatase, the changing pattern of activity suggested that the enzyme was partly derived from other sources, the most likely source being macrophage secretion. In addition, many of the vitreous macrophages contained partially digested endocytosed material, which has been described as a prerequisite for the induction of lysosomal enzymes, particularly acid phosphatase[16].

Although both extracellular and intracellular red cell lysis were seen to occur in the vitreous, the prolonged survival of a proportion of the red cells suggested that these cells were able to adapt to the unfavourable milieu and that inflammatory cell activity was insufficient to remove the red cells completely.

In addition to haemolysis, fibrinolysis occurred, albeit slowly, during the process of vitreous clot removal. The appearance of the clot after four weeks, as a fibrin skeleton devoid of red cells, has already been documented (see Chapter 12). Scanning electron microscopy of this material revealed an unusual honeycombed appearance (Fig. 13.20) and it is suggested that this structure was formed by cytoplasmic membranes of collapsed red cells filling the interstices of the fibrin network. Although fibrin deposits of this nature generally attract a polymorphonuclear response[434], they failed to do so in the vitreous (see Chapters 12,14). Since tissue fibrinolytic activity of the vitreous is low (see Chapter 7), fibrin removal is probably achieved by other means such as macrophage activity, particularly as elicited macrophages

are known to secrete plasminogen activator[516]. The delayed removal of fibrin probably reflected the low-grade mononuclear response[3].

The progressive changes in the structure of the clot and its components were accompanied by marked changes in the gel structure of the vitreous. The formation of vitreous "membranes" during vitreous clot resolution commenced with detachment of the vitreous from the retina, a process similar in ultrastructural appearances to that described for age-related posterior vitreous detachment[111,146]. This was followed by condensation of vitreous collagen fibrils, particularly around the clot, and resulted in loss of the gel state of the vitreous, and the formation of dense bundles of parallel, interwoven fibres. Such acellular "membranes" differed from cellular membranes which consisted of collagen-coated macrophage clumps, whose morphology changed during the course of time. Acellular membranes predominated in the later stages of clot resolution, when vitreous detachment was complete and their structure was similar to pelleted collagen from normal rabbit vitreous after centrifugation at 30,000G[492]. No fibroblasts were observed, nor was any 640A^{o} banded collagen detected, in contrast to the vitreous membranes which occurred after the intravitreal injection of autologous fibroblasts[3].

The pathogenesis of vitreous detachment in this condition is not known, but since its commencement coincided with the peak cellular influx at about 5 days (see Chapter 12) rather than with the physical trauma associated with the experimental technique, it is possible that this process also resulted from macrophage lysosomal enzyme secretion, as is suggested for the processes of haemolysis and fibrinolysis described above. Macrophages are known to secrete collagenase at neutral pH in vitro[60,551], and this may initiate the process by disrupting the fine three-dimensional collagen matrix[22]. The removal

of interfibrillar collagen bridges would have destabilised the network and permitted the individual fibrils to approximate each other, thus forming a dense collagen sheet. It is also possible that clot retraction exerted a physical influence on the surrounding collagen with which it was in intimate contact. However, in vitro studies did not support this concept (see Chapter 4).

The persistence of vitreous membranes within the vitreous is intriguing. Platelet-induced vitreous membranes are known to elicit only a moderate macrophage response[105], and the cellular response to vitreous haemorrhage is also reported to be low-grade (see Chapters 12, 14). The normal process of repair and remodelling by macrophages and fibroblasts which occurs when other connective tissue matrices are disrupted[70, 272] does not seem to occur so readily in the vitreous. This may in part be related to the high concentration of macromolecular hyaluronic acid which is known to modulate the behaviour of inflammatory cells considerably[24,147,449]. Similar mechanisms may be operative in the persistence of intact, healthy red cells within the vitreous for many weeks or months after haemorrhage.

CONCLUSION

In this chapter, the processes of haemolysis and vitreous membrane formation during vitreous clot lysis were studied in the rabbit eye by transmission and scanning electron microscopy. Haemolysis in the vitreous was predominantly an extracellular process, perhaps as a consequence of the unfavourable microenvironment. Macrophage activity was directed at clearing red cell debris.

Two forms of vitreous membrane developed during vitreous clot lysis, i.e. cellular and acellular. Neither of these were associated

with fibroblastic activity. Instead, they were composed of condensed vitreous collagen fibres. This evidence supports the view that fibroblastic activity within uncomplicated vitreous heamorrhages is an uncommon occurrence and that haematogenous vitreous membranes are composed of endogenous vitreous collagen with variable quantities of red cell debris and inflammatory cells.

CHAPTER 14

ELECTRON MICROSCOPY OF VITREOUS CLOT LYSIS: CELLULAR RESPONSE TO BLOOD IN THE VITREOUS

INTRODUCTION

Blood within the vitreous cavity elicits a well-defined inflammatory response[194,224,369], but it has been suggested in this thesis that the degree of cellular activity is insufficient to remove the blood clot completely (see Chapter 12). In the few morphological studies which have described the early phase of the inflammatory response to vitreous blood[194,369,486], significant cellular invasion of the vitreous was not observed until two or three days had elapsed[194], and only became fully established between the fifth and seventh day (see Chapter 12). This was in contrast to the brisk acute inflammatory response which ensued in experimental blood clots in other connective tissues[52,93]. In the vitreous, the cellular response to vitreous clots is predominantly mononuclear from the onset[194] (see Chapter 12). It thus appeared that the initial phase of marked polymorphonuclear cell migration which occurred in experimental thrombi in blood vessels[175,211] and in fibrin clots in the skin[389,434] had been suppressed. Furthermore, it has been suggested that the delayed clearance of fibrin from the vitreous during the course of reabsorption of vitreous clots may have been due in part to the lack of an adequate polymorphonuclear cell reaction (see Chapter 12). Other workers have reported a similar poor cellular response to platelet-fibrin deposits in the vitreous[106,160].

The mechanisms responsible for the poor cell response to blood in the vitreous are not known. It is possible, however, that the inflammatory response is modified by the specialised nature of the vitreous connective tissue matrix, which differs from other intercellular matrices in several respects (see Chapter 1). For instance, the resting cell content of the vitreous is extremely low, being re-

stricted to a single monolayer in the peripheral cortex of the gel[496] and it is not known what proportion of these cells represent true tissue macrophages. It is likely that few, if any, of these cells participate in the inflammatory process. The collagen content of the vitreous is also considerably lower than in other tissues, and vitreous collagen fibrils are extremely fine and have a particular spatial orientation (see Chapter 1). Such fibrils may provide an unfavourable surface for the migration of inflammatory cells. The absence of blood vessels in the vitreous may also influence the cell response by reducing the availability of plasma factors, such as opsonins, which are required for the full expression of the inflammatory response[448]. Furthermore, it has been proposed that the glycosaminoglycan composition of the vitreous gel, which is predominantly hyaluronic acid with minimal sulphated proteoglycans[491] may have an inhibitory effect on cell influx into the vitreous, particularly since hyaluronic acid in high dosage has been shown to suppress leukocyte motility in vitro[24]. Other factors may contribute to the inadequate cell response to vitreous blood, such as absence of appropriate chemotactic stimuli (Chapter 9).

The ultrastructure of phagocytic cells in inflammatory exudates has been studied in several tissues[52,184,387,444], but there have been no similar reports of inflammatory cell responses in the vitreous. In view of the unusual nature of this response, as shown by light microscopy, the present ultrastructural study was undertaken. Scanning electron microscopy was also used since it was considered that this was a unique opportunity to study the surface morphology of inflammatory cells in an atypical connective tissue matrix.

MATERIALS AND METHODS

The materials and methods used for the study of the inflammatory cell response to vitreous blood were the same as those used for the studies described in Chapter 13, since both studies formed a part of the same ultrastructural investigation. A full description of the methods and materials for this part of the thesis is given in Chapter 11, including the methods for inducing vitreous clots in the rabbit eye and for the dissection and preparation of tissue for ultrastructural analysis.

ANIMALS

Sixteen mature New Zealand white rabbits were used in this study. The following time periods provided material for examination: one day, three days, six days, three weeks, six weeks, nine weeks, twenty weeks, and eighteen months after injection of blood into the vitreous. Two animals were killed at each of these times.

RESULTS

There was little evidence of cellular invasion into the vitreous cavity twenty-four hours after the introduction of whole blood. The clot, which formed immediately after the injection of blood into the vitreous, remained as a discrete mass within a normal vitreous gel. Some changes in the shape and electron density of the red cells within the clot were found and a few red cells showed cytoplasmic blebs and vacuolar changes (Fig. 14.1a). The surface of the clot comprised a

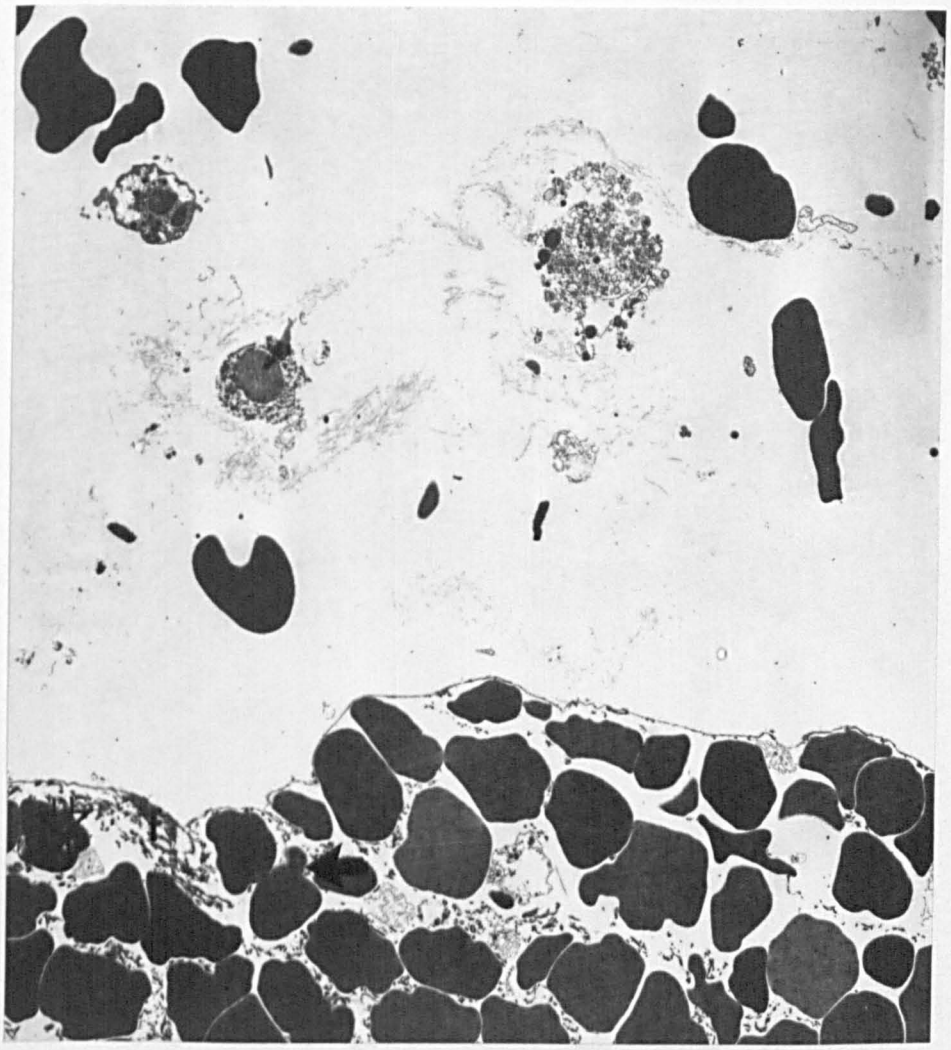

Figure 14.1 Twenty-four hour vitreous clot, T.E.M.

(a) clot, in lower half of the field, shows masses of relatively normal red cells. There is some variation in electron density of the red cells, plus blebbing of cytoplasm (arrow) and formation of small vacuoles (arrow head). A single membrane surrounds the clot. Fibrin (f) and degranulated cell forms (presumably platelets) are also present. Three polymorphonuclear leukocytes are seen in the upper field, in advanced stages of degeneration and surrounded by fibrillar material. x 2,900.

single continuous membrane which enclosed both the red cells and fibrin deposits (Fig. 14.1a). Fibrin was not found outside this layer. A few polymorphonuclear leukocytes were dispersed randomly throughout the clot and its immediate vicinity (Fig. 14.1a). These cells were in various stages of cytolysis, as seen by their extreme cytoplasmic rarefaction and organelle disruption (Figs. 14.1a,14.1b). They were generally surrounded by fine unbanded fibrils which presumably were vitreous collagen. There was little evidence of active phagocytosis by these cells.

After three days, few changes had occurred in the clot itself; in particular, there was no evidence of inflammatory cell invasion of its structure. Some young mononuclear cells with a thin rim of cytoplasm were seen, initially surrounding the ciliary processes and later within the vitreous gel. By the sixth day, these cells were more numerous, but were still relatively immature, as shown by the prominent margination of the nuclear heterochromatin, the moderate nucleus to cytoplasm ratio, and the paucity of cell organelles (Fig. 14.2). They were intimately associated with surrounding fibrillar material and small electron dense particles. Acid phosphatase activity within these cells was minimal.

Scanning electron microscopy showed that the surface of the clot after six days had acquired a loose network of strands and fibres which formed an incomplete sheet (Fig. 14.3). This represented a considerable thickening of the single layer which surrounded the clot at twenty-four hours. However, the six-day-old clot was still intact and showed little evidence of undergoing clot lysis. When the clot was viewed at higher magnification, moderate numbers of single rounded macrophages were found on its surface (Fig. 14.4a). These cells varied considerably in size, and they possessed well developed ruffled plasma membranes and elongated

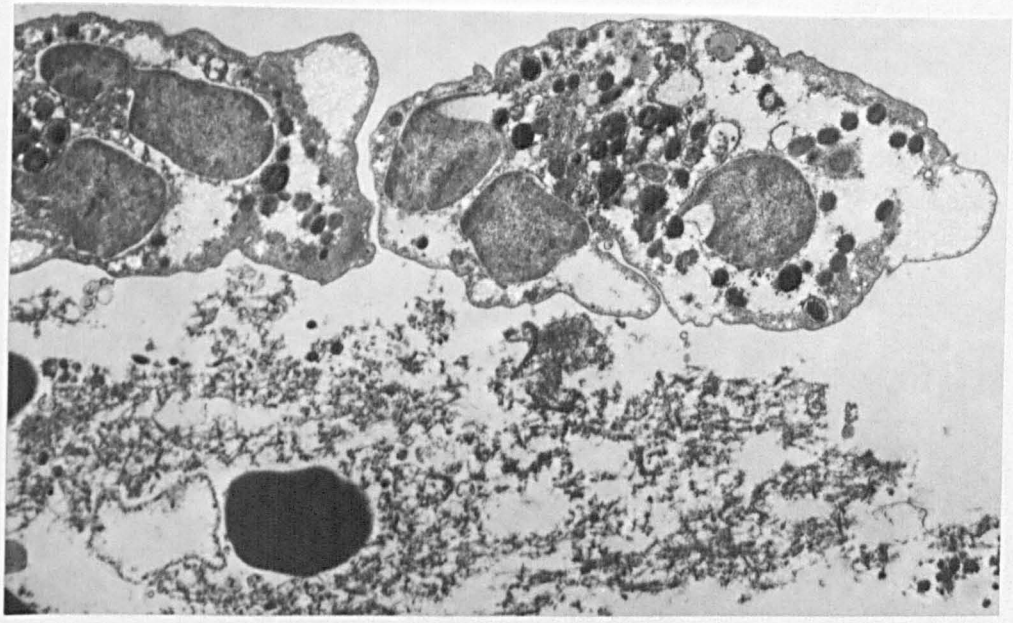

Figure 14.1 Twenty-four hour vitreous clot, T.E.M.

(b) higher power view of two polymorphs within the clot, showing cytoplasmic rarefaction and organelle disruption. x 4,400

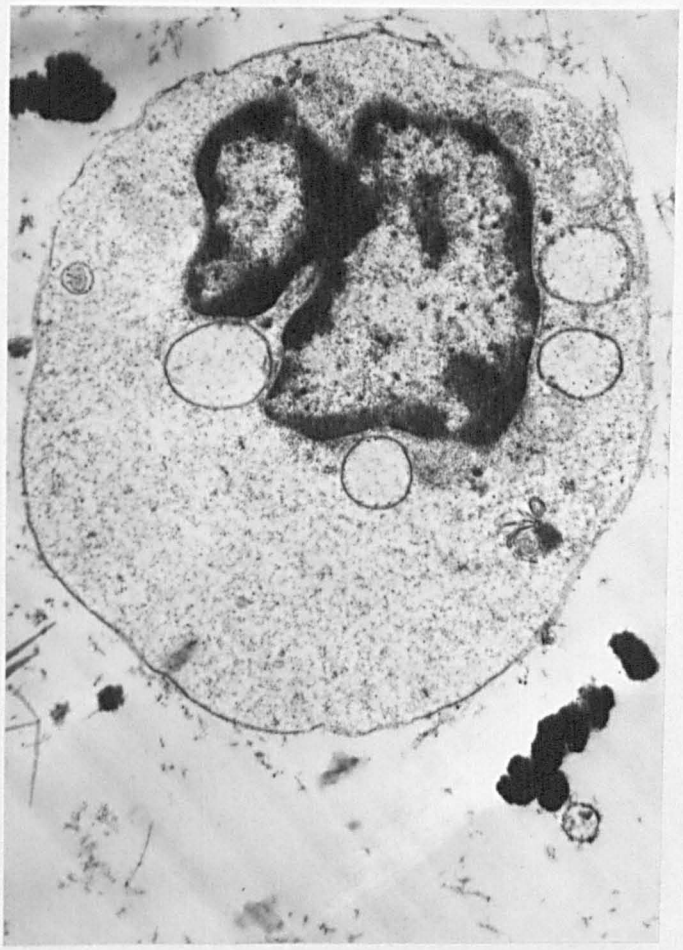

Figure 14.2 Six day vitreous haemorrhage, T.E.M. Young macrophage within the vitreous, near vitreous base. Nucleus shows prominent margination of the heterochromatin. Organelles are few; no primary lysosomes or phagosomes are seen. x 9,900

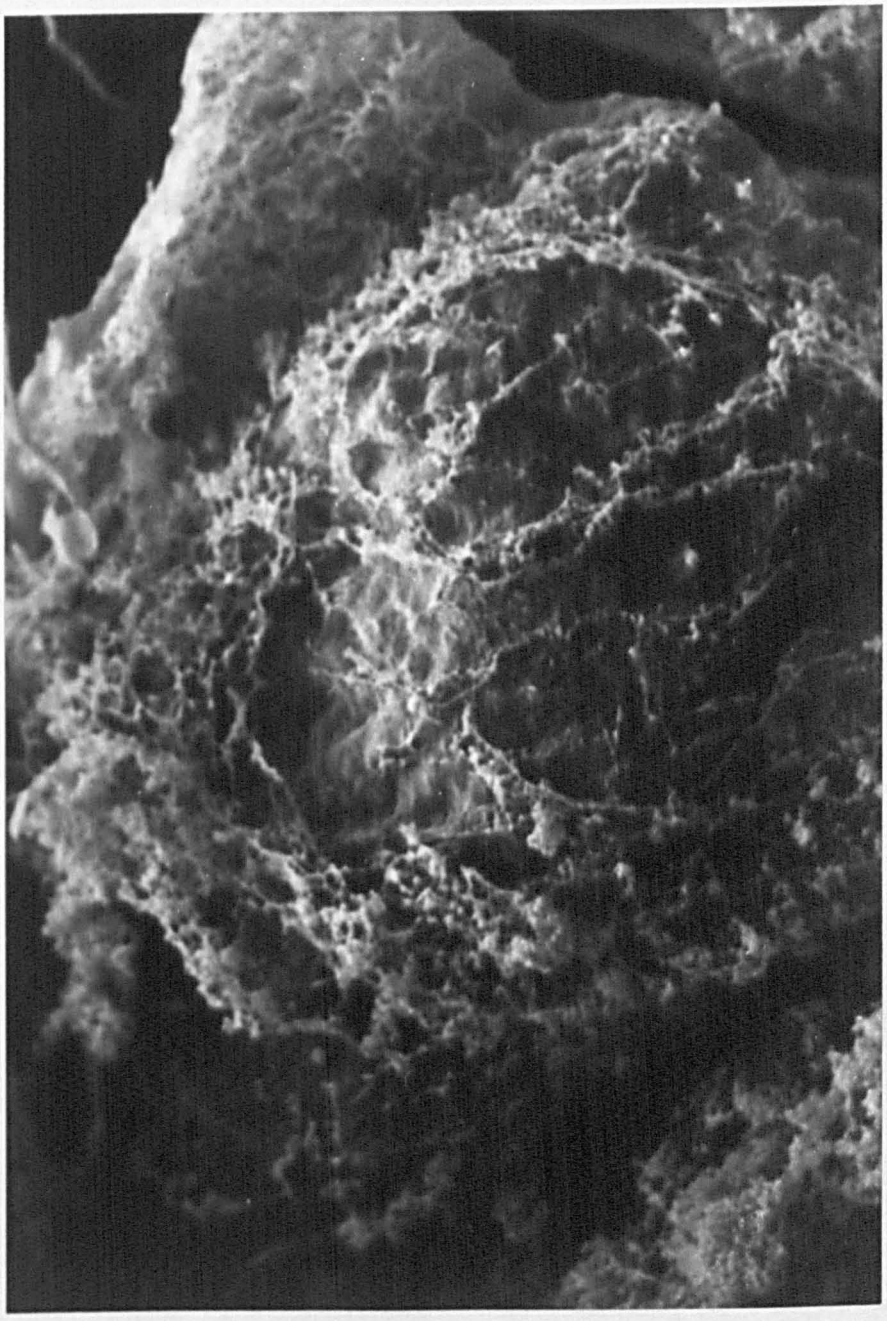

Figure 14.3 Six day vitreous clot, S.E.M. Surface of vitreous blood clot is composed of a loose network of strands and fibres forming an incomplete sheet. x 180.

microvilli (Fig. 14.4b). It was unusual, however, to find spread cells or cells with obvious lamellipodia. The clot surface on which these cells rested was composed of a complex lattice of smaller fibres. Most of the holes in this fine grid were devoid of electron dense material, but some contained variably-sized round bodies and other amorphous debris. Few intact red cells were seen at this surface level.

From this stage onwards, the macrophage population increased slowly until by three weeks the vitreous contained substantial numbers of large cells showing numerous granules within their cytoplasm (Fig. 14.5a). The granules varied in size and density, often having a diameter of more than 30 ,um. Thick section analysis also suggested that some of these cells were multinucleate (Fig. 14.5a). Electron microscopy indicated that the granules in these cells were phagocytic inclusions, or secondary lysosomes, which almost filled the cell cytoplasm (Fig. 14.5b). There was marked variation in electron density, size and shape of the inclusions; some showed a homogeneous dark matrix, whereas others had a highly variegated appearance with a granular background (Fig. 14.6a,14.6b). Several myelin whorls were seen and some cells contained numerous lipid droplets (Fig. 14.6a). Occasionally, a recently ingested intact red cell was found within the macrophage cytoplasm (Fig. 14.6a). Electron-lucent vacuoles and micropinocytotic vesicles were commonly observed, and less frequently, dilated intracytoplasmic channels, which suggested intracellular oedema (Fig. 14.6b). Acid phosphatase activity varied within the phagosomes, but was especially marked in primary lysosomes at this stage (Fig. 14.6b). Occasionally, a primary lysosome was found in close association with a phagosome, which gave the appearance of fusion of the two organelles (Fig. 14.6b).

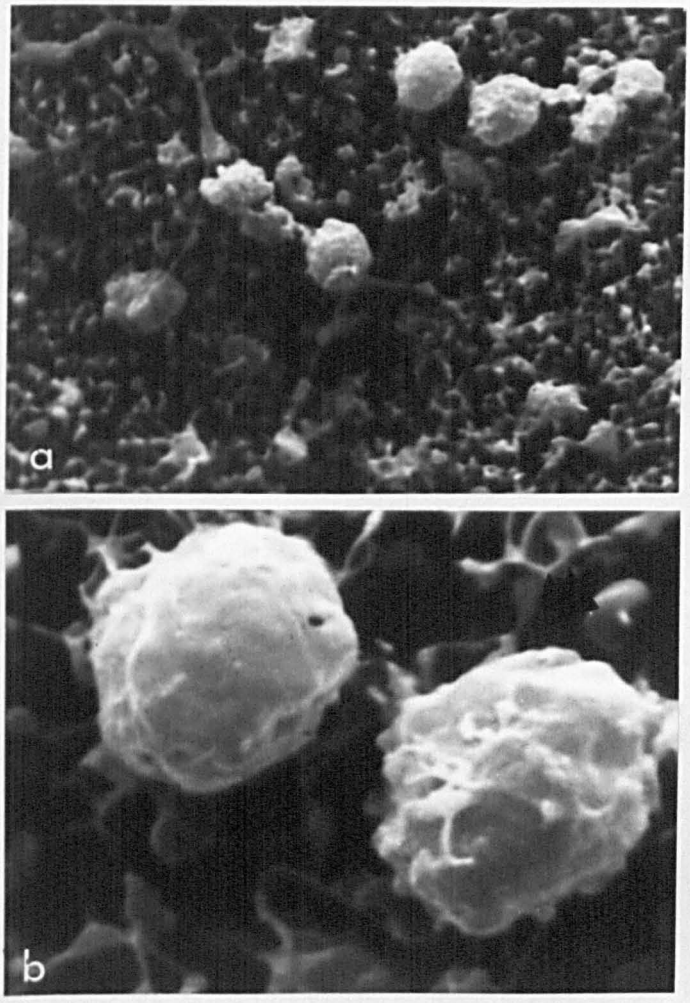

Figure 14.4 Six day vitreous blood clot, S.E.M.

(a) Cells on clot surface are single and rounded, and vary in size. The substratum is composed of complex lattice of small fibres. x 500

(b) Higher power view of Fig. 4(a) showing ruffled plasma membrane and microvilli. A single red cell is seen in the background (arrow). x 1,200

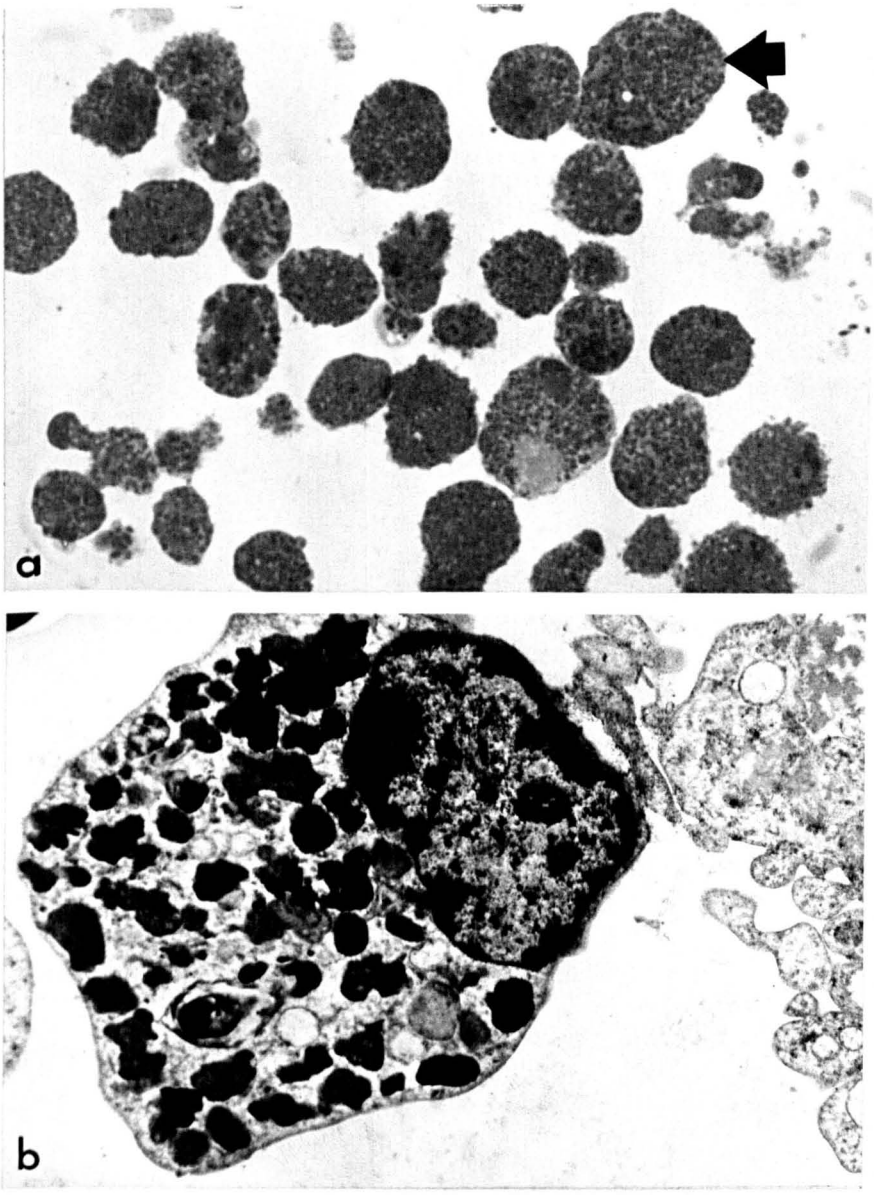

Figure 14.5 Three week vitreous clot.

(a) Thick section light microscopy. The cells are very large and have a densely granular cytoplasm. Some cells appear to be multinucleate (arrow). x 450.

(b) Transmission electron micrograph of macrophages within the vitreous. The cytoplasm is filled with phagosomes which show great variation in shape and electron density. Eccentrically placed nucleus contains two nucleoli. x 6,500.

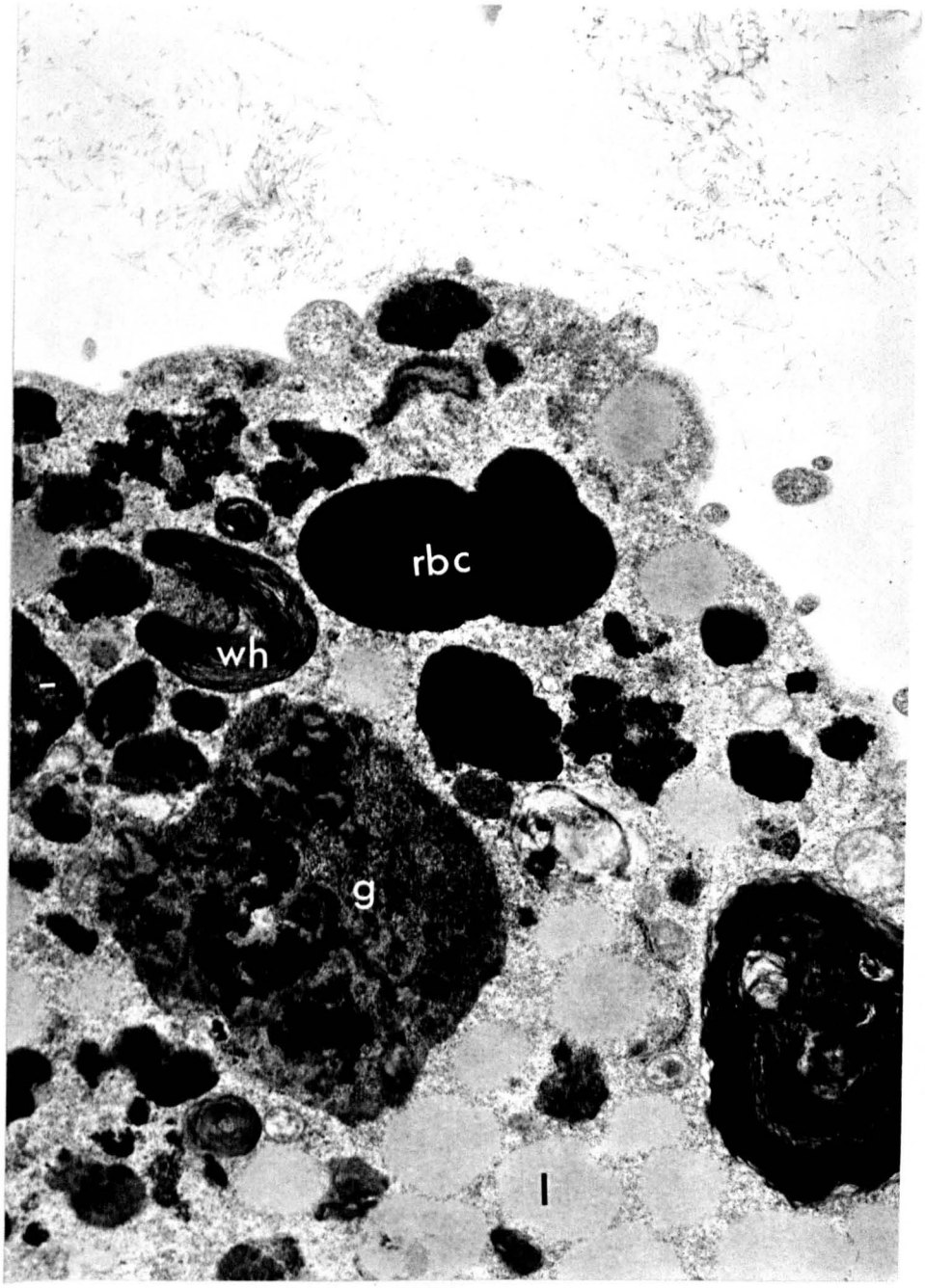

Figure 14.6 Three week vitreous haemorrhage, T.E.M.

(a) Marked heterogeneity of the macrophage cytoplasmic inclusions is shown. Intact red blood cell (rbc), myelin whorl (wh), lipid droplets (l), granular inclusion (g). x 13,600

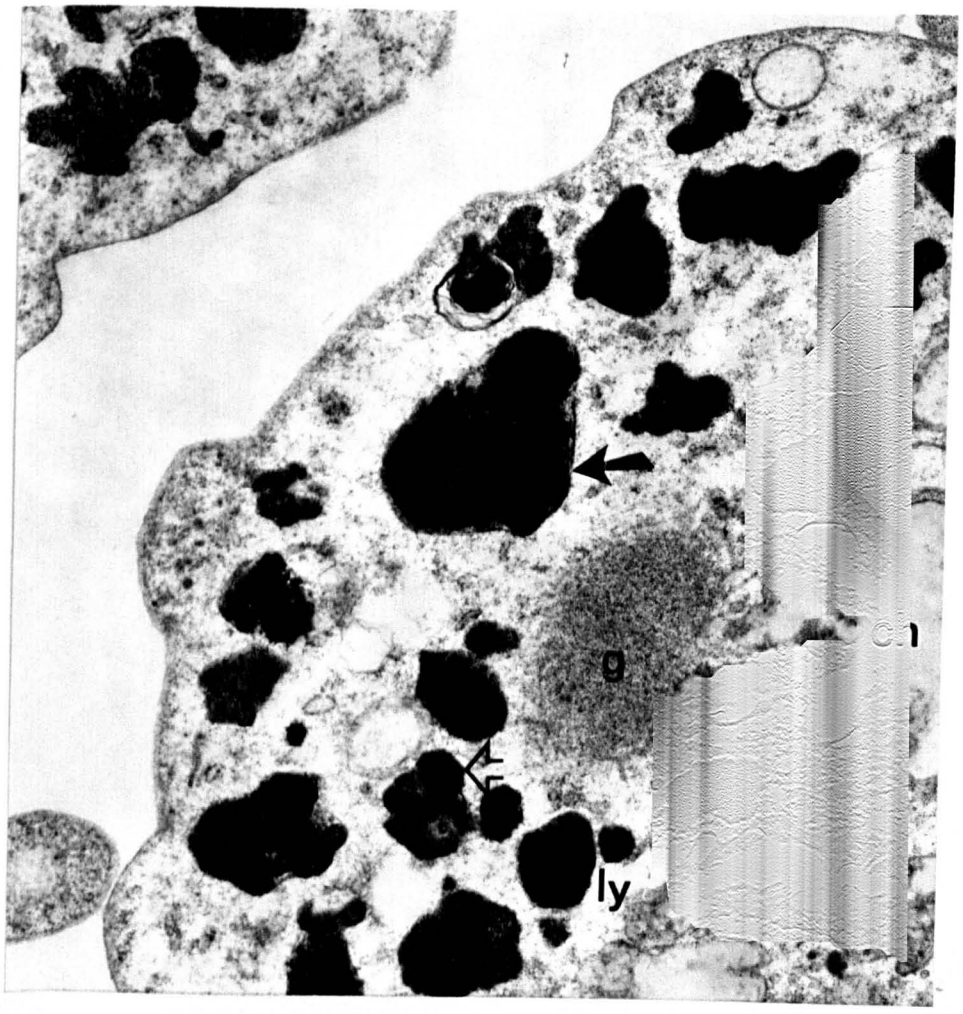

Figure 14.6 Three week vitreous haemorrhage

(b) Acid phosphatase staining of inclusions is especially marked in primary lysosomes (ly). Intracytoplasmic channels (ch), membrane bound phagosomes (arrow), granular inclusion (g). Apparent fusion of primary lysosomes with phagosomes is observed (arrow head). x 18,800

Several secondary lysosomes were enclosed in a single or double membrane, but in others it was difficult to identify a membrane.

Few other organelles were identified in these large cells, such as mitochondria, endoplasmic reticulum or Golgi complexes. The eccentrically placed nucleus often showed one or more nucleoli and margination of nuclear heterochromatin (Fig. 14.5b).

Between three and six weeks after induction of the vitreous clot, several morphological changes occurred in the cell exudate. A striking feature was the formation of massive cell aggregates (Fig. 14.7a) which occurred in various loci within the vitreous. Since the main clot structure had disintegrated by this stage (see Chapters 12,13), it is possible that these large cell clumps represented fragments of the original clot. The cells in these clumps were uniformly very large, sometimes with a diameter of more than 100 μm, and between the cells lay small accumulations of amorphous debris (Fig. 14.7a). Fine fibrous strands radiated from the clumps of cells, but it was difficult to identify the nature of the substratum to which the cells adhered. It is likely that this layer was composed of residual clot debris. The surface morphology of the cells at this stage was notably uniform. They were generally rounded or oval, and smooth-surfaced, and where microvilli were present, these were short and thick (Fig. 14.7b). Some cells showed pits and indentations, measuring 0.1-2 μm, of their surface membranes, while other cells had a complex system of ridges, flaps and folds. Filiform extensions of the peripheral cytoplasm were seen in only a few cells, and it was rare to find distinct lamellipodia. Several cells showed prominent bulges of the surface membranes, while others had a cobblestone appearance (Fig. 14.7b).

Between six weeks and eighteen months after the injection of blood into the vitreous, total cell numbers gradually decreased, but their

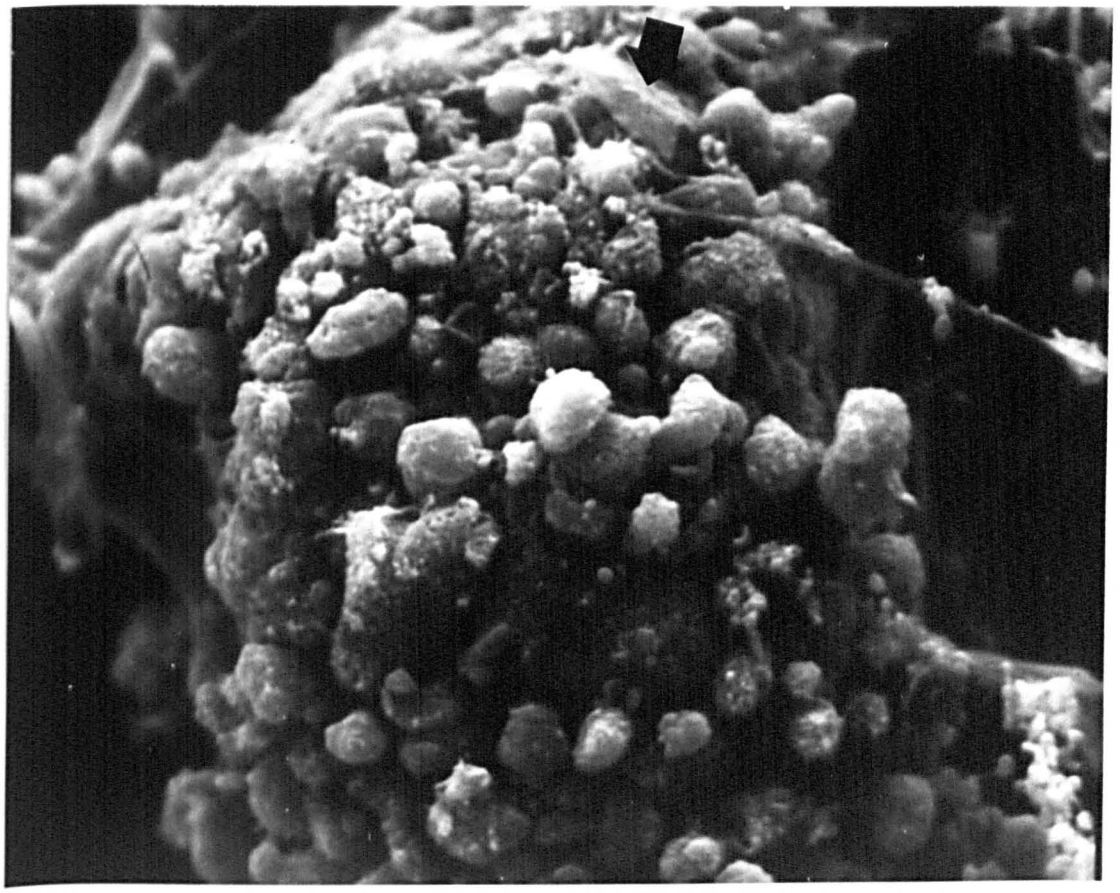

Figure 14.7 Six week vitreous haemorrhage, S.E.M.

(a) Aggregate of cells. The cells are very large and generally rounded. Amorphous debris noted between the cells. A fibrous strand is observed attached to the cell mass. A single spread cell is seen at the top of the field (arrow). x 600.

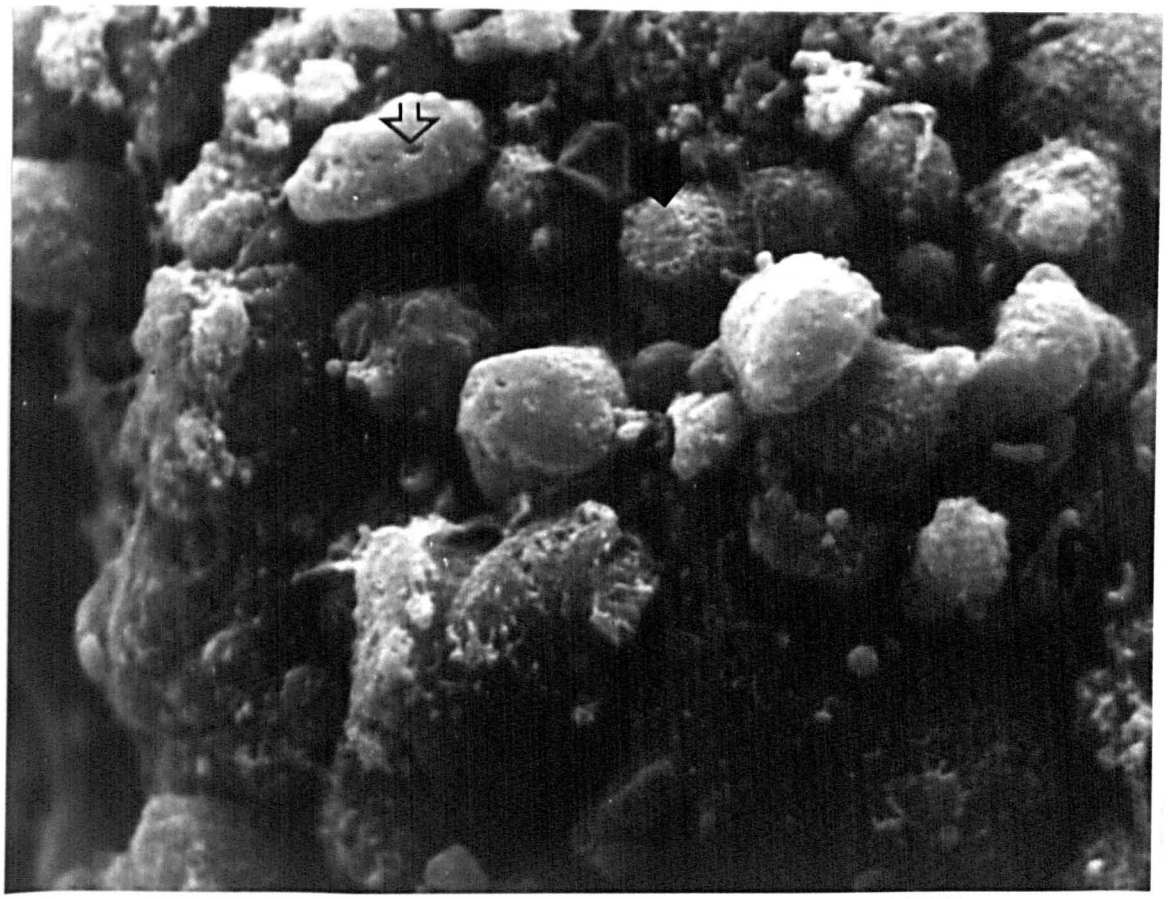

Figure 14.7 Six week vitreous haemorrhage, S.E.M.

(b) The cell surface is variably smooth or cobblestone. Microvilli are short and thick when present. Some cells have filiform extensions (arrow). Pits and indentations are seen in other cells (arrow heads). Occasional intact red cells are present in the cell mass (curved arrow). x 900

morphology was similar to that at six weeks. Most of the cells were very large, sometimes measuring more than 200 μm in diameter, and they contained very large numbers of phagosomes similar to those described above (Fig. 14.8a). However, some cells with prominent microvilli contained numerous small (3 μm) round inclusions of uniform size, whereas the inclusions in other cells were extremely variable in size. These latter cells also had fewer microvilli (Fig. 14.8a). Acid phosphatase activity within the cells also varied, and was most marked in those cells with the smaller inclusions (Figs. 14.8b and inset). These appearances suggested that there was considerable variation in the phagocytic capacity of the cells. In addition, electron dense material, which closely resembled the intracellular inclusions, was frequently encountered in the extracellular space (Fig. 14.9), particularly in association with healthy macrophages. This suggested that some of the cells had undergone cytolysis with release of their cell contents which were then rephagocytosed by other active cells (Fig. 14.9).

Few other cell types were observed. The proportion of young cells in the cell exudate was low after three weeks, although fresh cells were found even in the late stages of blood clearance (Fig.14.10a). It was more common, however, to find multinucleate giant cells, usually as members of a large cell aggregate (Fig. 14.10b). These cells contained numerous phagolysosomes, similar to those in conventional macrophages; their nuclei often showed one or more nucleoli and a thin rim of nuclear heterochromatin. Primary lysosomes were few. There appeared to be segmental condensation of the cytoplasm in areas where there was intimate contact between the cell membranes of two cells, but no fusion of cell membranes was seen between mature cells (Fig. 14.10b). Only when young cells were present in association with large

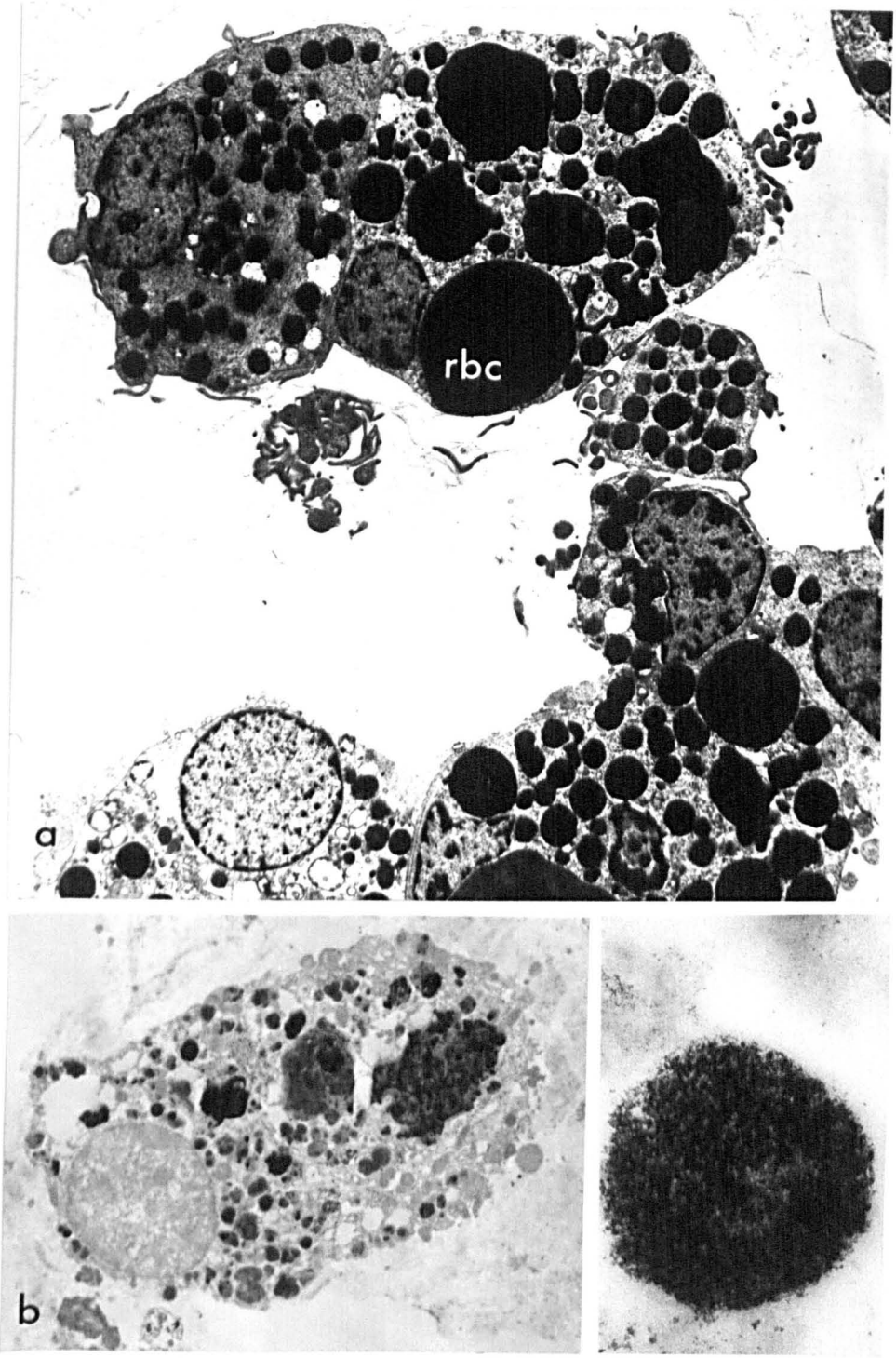

Figure 14.8 Nine week vitreous haemorrhage, T.E.M.

(a) Aggregate of macrophages. Inclusions are similar to those seen earlier. However, some cells show uniform small inclusions, while others show marked variation in size of inclusions. Recently ingested red cell (rbc). x 3,800

(b) Marked acid phosphatase reaction product in a macrophage with small inclusions. x 3,700.

Particularly high activity was observed in primary lysosomes (inset). x 90,000

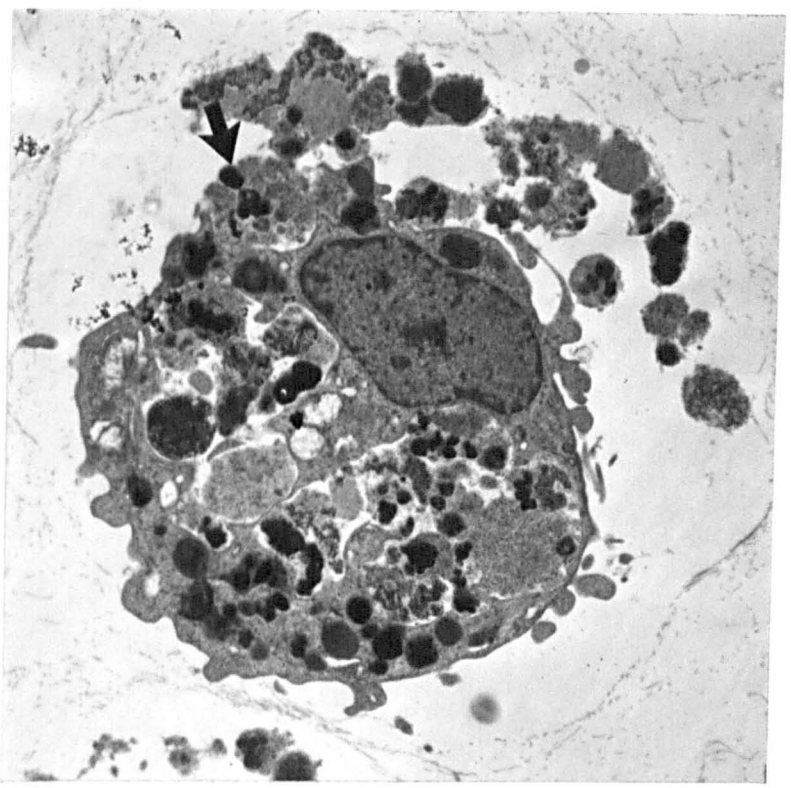

Figure 14.9 Twenty-week vitreous haemorrhage, T.E.M.

Considerable amorphous debris is noted in the extracellular space surrounding an active vitreous macrophage. This material is similar to the cytoplasmic inclusions, and appears to be undergoing phagocytosis (arrow). x 3,800

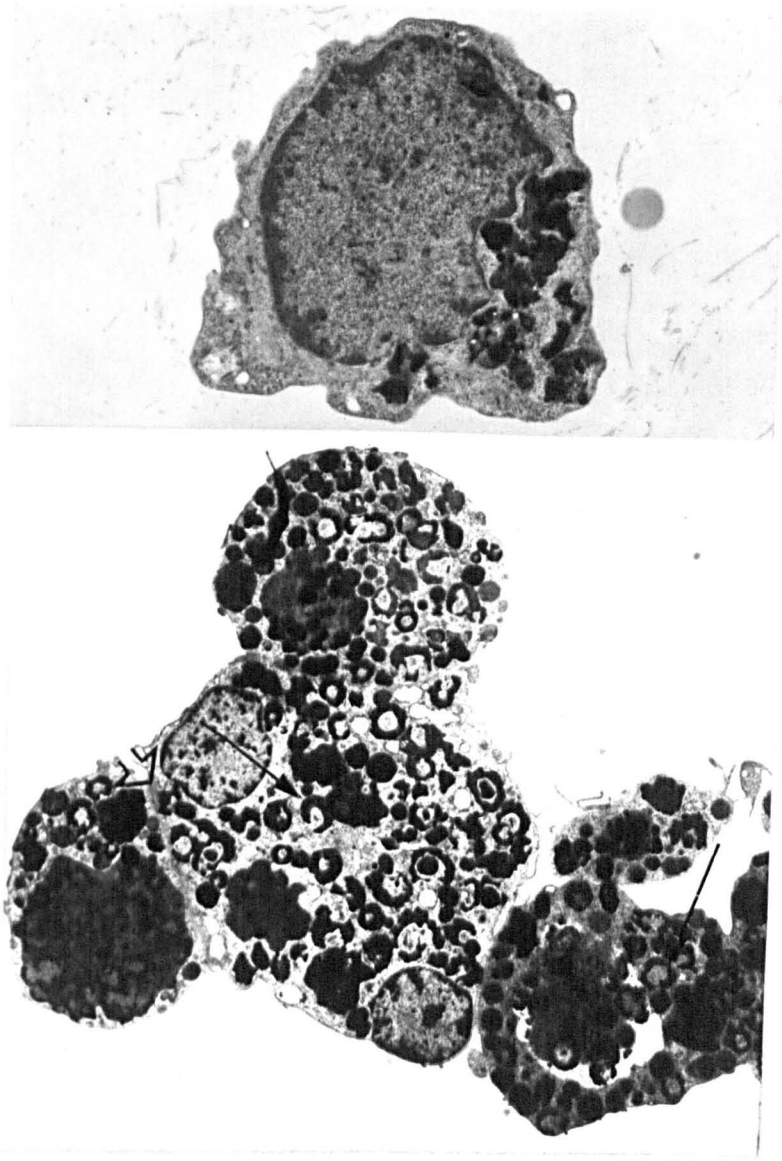

Figure 14.10 (a) Young vitreous macrophage eighteen months after injection of blood into the vitreous. The cell shows a high nucleus to cytoplasm ratio, and contains few phagosomes. x 6,400.

(b) Cell aggregate twenty weeks after injection of blood into the vitreous. A large multi-nucleate cell is shown. Tunnelisation (see discussion) is particulary well seen in these cells as sickle-shaped and ring-like inclusions (arrows). Condensations of cell membrane are seen in areas of close contact between cells (arrowhead). x 2,800.

mature macrophages was fusion of the plasma membranes suggested by the bridging of cytoplasmic contents between the two cells (Fig. 14.11).

The cellular response to blood in the vitreous was therefore composed largely of mature macrophages, and to a lesser extent, multinucleate giant cells and young round cells. Epithelioid cells and fibroblasts were not seen. Some of the round cells could not be distinguished from lymphocytes by morphological means, but their numbers were few after three weeks.

DISCUSSION

Previous studies of the inflammatory response to vitreous blood clots have described the slow emigration of cells into the vitreous[194] and the relative lack of polymorphonuclear leucocytes within the initial exudate[194] (see Chapter 12). These findings have been confirmed in the present study. The few polymorphonuclear cells which were seen twenty-four hours after the injection of blood into the vitreous occurred within and around the original clot, and probably represented cells which had been introduced with the blood at the time of injection. This was further suggested by their advanced state of degeneration and their absence of active phagocytosis. Alternatively, if these cells did correspond to the initial phase of polymorphonuclear leucocyte emigration, their numbers were considerably fewer than was reported in the inflammatory exudates to similar blood clots in the vascular tree[175,211] or dermis[52], or in fibrin clots in the skin[389,434]. The central role of polymorphs in the clearance of fibrin deposits in inflammatory exudates has been amply demonstrated[48,389,405], and this mechanism, in association with the intrinsic fibrinolytic activity of most tissues (see Chapter 7) provides a means of rapidly clearing

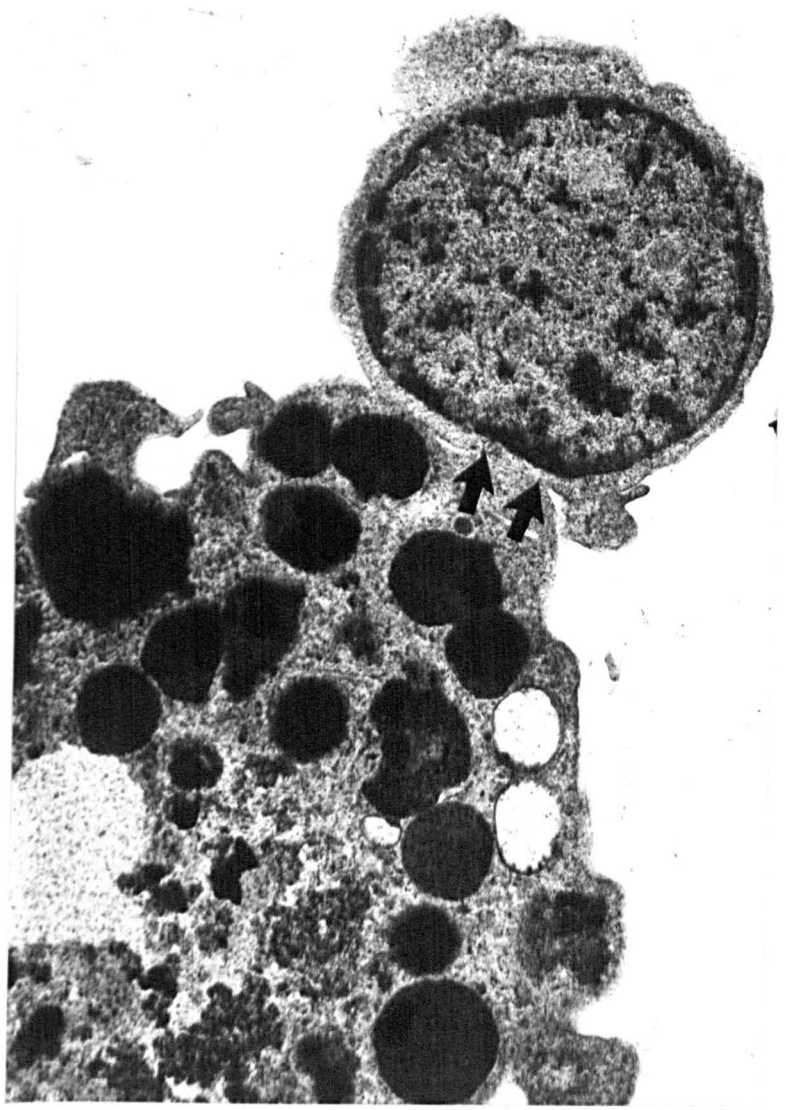

Figure 14.11 (a) Cell exudate twenty weeks after blood injection into the vitreous. An immature round cell is seen in close association with a mature macrophage. Arrows indicate an area of possible fusion of cell membranes. x 12,300

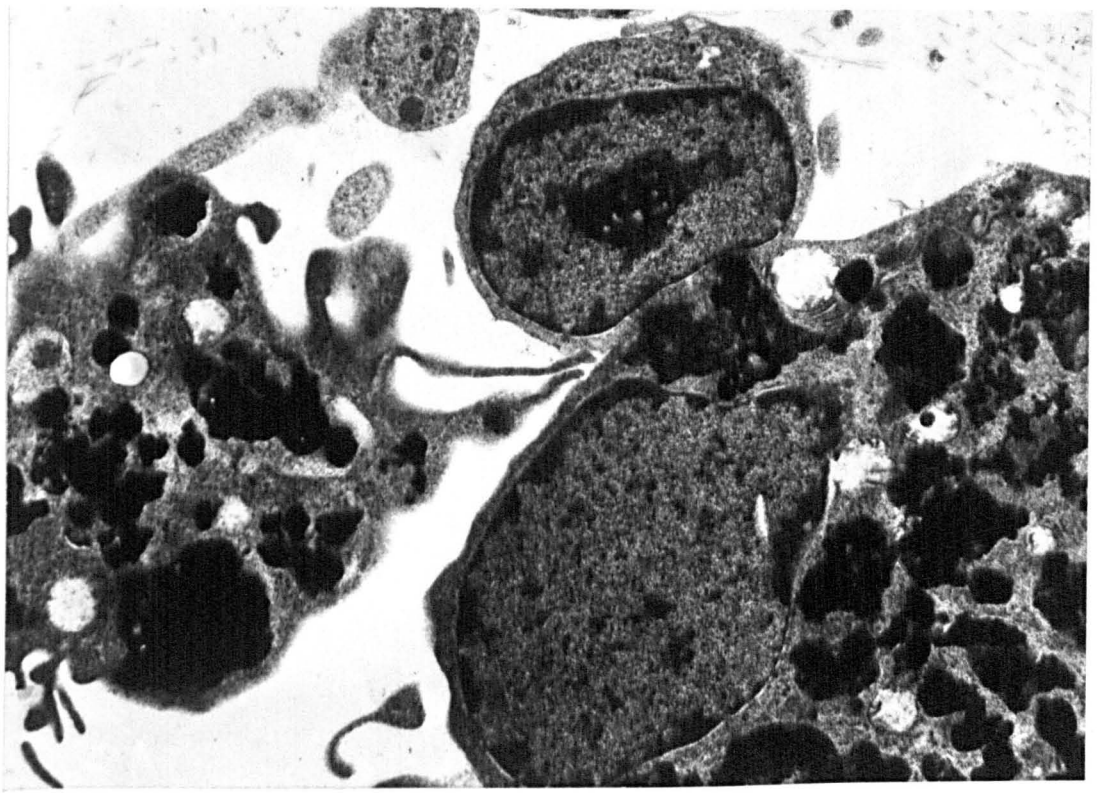

Figure 14.11 (b) Cell exudate eighteen months after blood injection into the vitreous. Fusion of cell membranes between a young mononuclear cell and a mature macrophage is suggested by bridging of cytoplasmic contents between the two cells. x 10,900

fibrin from injured tissues and ensuring a return of structural integrity. Indeed, within hours of placing fibrin clots on cover slips into the skin of rabbits, Riddle and Barnhardt[389] observed massive accumulation of polymorphonuclear leucocytes, not only surrounding the clot, but invading its depths and forming pockets within its substance. Clearly, the response to blood clots in the vitreous was different. Penetration of the blood clot by inflammatory cells did not occur, and indeed the clot remained as a discrete mass for at least four weeks.

The low levels of tissue fibrinolytic activity in the vitreous (Chapter 7) and the failure of vitreous blood to generate an adequate polymorphonuclear cell response both probably militate against the removal of blood clots from the vitreous. Even after one month, when most of the red cells had either been lysed or had diffused away from the clot (see Chapter 12) a fibrin framework of the original haematoma remained. Additional biochemical evidence has shown that up to six weeks elapsed before fibrin was removed from an experimental vitreous clot (see Chapter 8). It would thus appear that both the inadequate polymorphonuclear cell response and the low fibrinolytic activity of the vitreous contributed to this delayed fibrin clearance.

The inflammatory response to blood in the vitreous has thus been shown to be predominantly mononuclear. Mononuclear cell invasion was observed seventy-two hours after blood injection and gradually increased until the third week. After this time, fresh cells were infrequently observed, and the lesion consisted of mature macrophages and multinucleate giant cells in large cell aggregates. The small numbers of fresh cells, and the characteristic ultrastructure of the long-lived macrophages, namely masses of ingested insoluble material within the phagosomes and paucity of primary lysosomes, were similar to the

appearances of cells in a "low-turnover"granuloma[387].

The insoluble material within the cells represented partially degraded red cells which showed all stages of lysis from recently ingested erythrocytes to myelin whorl formation (Fig. 14.6c). This process of intracellular haemolysis in alveolar macrophages has been fully documented by Collett and Petrik[103,104]. Both the granular and the haemolytic pathways of red cell breakdown recently described by Ziligs[575] were noted in vitreous macrophages, although the former was much more common. In several cells, however, the degradation process seemed to have ceased at the stage of "tunnelisation", a term coined by Edwards and Simon[131] to describe the inspissation of phagocytic cell cytoplasm into the erythrophagosome (Fig. 10b). Crystalline structures within the inclusions were rare. The digestive capacity of the cells varied and many cells appeared to be metabolically inert, although the contained numerous partly digested inclusions. Such cells probably underwent cytolysis with release of their cell contents into the extracellular space. These particles were then rephagocytosed by other cells.

It has been stated that the degradation of red cells in vivo occurs entirely within the cytoplasm of mononuclear phagocytic cells, following engulfment of the whole red cell[398]. This was shown to be true for the physiological removal of senescent red cells from the circulation by spleen macrophages[398] and for the removal of red cells from experimentally-induced blood deposits in the bronchial tree[103,104]. However, within the vitreous, in addition to the usual process of macrophage erythrophagocytosis, considerable extracellular lysis of red cells was observed (see Chapter 13). Such haemolysis began before the onset of cellular invasion, as shown by changes in electron density of the red cells and vacuole formation (Fig. 14.1a). Similar electron micro-

scopic observations of extracellular red cell lysis were noted in peritoneal blood clots[135].

The formation of multinucleate giant cells during the inflammatory response to vitreous blood is interesting for several reasons. Recent studies have suggested that the phagocytic ability of cell populations containing giant cells is lower than similar pure mononuclear cell populations[385]. Both the surface morphology and the ultrastructure of vitreous macrophages would support this concept, since their appearances were similar to other poorly phagocytic macrophages and giant cells[75,80,385]. In addition, the formation of multinucleate giant cells appeared to occur by the fusion of young cells to older mature macrophages (Fig. 11a,b). Support for this proposed mechanism of giant cell formation comes from the recent kinetic studies of Chambers[79]. Although macrophages and giant cells in inflammatory exudates are derived from precursors in the marrow[386,517], it has been suggested that the formation of one cell type is exclusive of the other[386]. It would therefore appear that not only is the cell response to blood in the vitreous atypical in terms of cell type and cell numbers, but the preferential formation of multinucleate giant cells and the appearances of the macrophages suggests that those cells which were engaged in vitreous red cell breakdown had impaired phagocytic ability.

There are several possible reasons for this unusual cell response to vitreous blood. During experiments on the mononuclear cell response to blood clots in the skin, Ghani[175] observed a similar pattern of cell activity, with the formation of large macrophages, epithelioid cells and giant cells, when the clot was sequestered in a millipore chamber. Thus, an artificial barrier to the invasion of inflammatory cells, but not to solutes or nutrients was created. A similar situation may obtain naturally in the vitreous where components of the connective tissue

matrix may provide a barrier to the ingress of cells. Thus, the high content of polydisperse hyaluronic acid in the vitreous may inhibit inflammatory cell movement in vivo, as it has been shown to do in vitro[24] (Forrester and Wilkinson, experiments in progress).

It is well established that modifications of the inflammatory process occur in various specialised tissues[541]. The present investigation of the cell response to vitreous clots provides a further example of this altered response and raises a number of interesting questions concerning the role of connective tissue components in the evolution of the inflammatory response.

CONCLUSION

This study of the inflammatory response to vitreous blood deposits has confirmed previous investigations which showed that the cell response was slow to develop, was predominantly mononuclear, and remained low-grade. The formation of large aggregates of giant macrophages and multinucleate cells was also observed. It is suggested that the atypical cell response to blood in the vitreous may be due to the unique nature of the vitreous as a connective tissue, particularly to its high content of hyaluronic acid, which has been shown to inhibit inflammatory cell activity in vitro.

In addition, the scanty cellular response to blood in the vitreous may be a contributory factor to the delayed resolution of intravitreal haemorrhages.

APPENDIX

CHAPTER 15

THERAPEUTIC CONSIDERATIONS IN VITREOUS HAEMORRHAGE

INTRODUCTION

Since vitreous haemorrhage was first recognised as a cause of blindness, its treatment has proved difficult. Spontaneously resolving small vitreous haemorrhages usually require no intervention. On the other hand, the problem of the dense and persistent vitreous clot has brought forth a multitude of clinical and experimental methods designed to promote its reabsorption. Indeed, Jaffe[237] pointed out that the wide variety of treatments for this condition attested to the inefficacy of most of them. Until recently, the common approach to vitreous haemorrhage was conservative, and only in rare cases was any surgical intervention, such as cryotherapy or vitreous aspiration, contemplated. Consequently, numerous patients were permanently blinded by this condition. However, the explosion of interest in surgical techniques of vitreous removal and replacement has altered radically the approach to the patient with a vitreous clot.

The purpose of this chapter is to view the basic pathophysiological studies described in the previous chapters against the background of current trends in therapy. Three therapeutic approaches to vitreous haemorrhage have been adopted which involve the use of surgical, biophysical or clot-lysing techniques.

SURGICAL TECHNIQUES

A surgical approach to opaque vitreous is not a new concept. Von Graefe [527] was the first to report on the surgical dissection of vitreous "membranes" and later, Ford[142] aspirated opaque vitreous. Ford's lead formed the basis of surgical intervention for more than half a century, with only minor modifications[51,579,580], particularly in relation to

replacement of the opaque vitreous[110]. Their failure to gain universal support was a reflection of the high incidence of complications and generally unfavourable results. Following the introduction of the operating microscope and intensive research during the 1950s and 1960s, pars plana vitrectomy became a practical procedure. This operation utilises a custom-built instrument, the vitreous infusion suction cutter (VISC) which requires the use of an operating microscope and corneal contact lens for vitreous surgery. Subsequently, a large number of vitrectomy instruments have been designed[122,156,256,269,301,375,393,511] and numerous papers, notably from the U.S.A. on the indications, complications, techniques and other aspects of vitreous surgery have appeared. From the beginning, it has been shown that simple vitreous haemorrhage is a firm indication for vitreous surgery. Machemer and Norten[302] reported that eighteen of twenty cases of simple vitreous haemorrhage gained some degree of visual improvement after vitrectomy. Follow-up of these cases ranged from one to twelve months. Diabetic vitreous haemorrhages with extensive proliferative retinopathy did less well, visual improvement occurring in sixteen of twenty-three cases (eighty per cent), while the presence of retinal detachment militated against successful surgery (two of eight cases). Similar findings were reported by Mandelcorn et al[309], who claimed a seventy-one per cent success rate for simple vitreous haemorrhage, but only a thirty-one per cent rate of improved vision in complicated vitreous haemorrhage. Peyman et al[394] reported an overall improvement in vision in sixty-eight per cent of cases with forty-five per cent gaining a vision of 20/300 or better. A more recent study by this group[395] reported similar figures of sixty-six per cent improvement for cases of simple vitreous haemorrhage and forty-two per cent for cases of vitreous haemorrhages complicated by retinal detachment.

A further large series was reported by Michels et al[332] with an overall success of sixty-five per cent, although a different method of visual acuity standardisation was used.

It is clear that a majority of patients with uncomplicated, non-resolving vitreous haemorrhage can expect some degree of visual recovery from this form of surgery. However, numerous complications associated with vitrectomy have become apparent following the publication of these series[302,309,332,295]. Complications may be either intraoperative or post-operative (Table A1). The incidence of major complications such as phthisis bulbi and rubeosis iridis varies from sixteen to twenty-five per cent. The risk of worsening the visual status has been emphasised by other groups, particularly among diabetics with proliferative retinopathy[374,572]. It is apparent, therefore, that while this form of treatment can afford enormous benefit to the patient, it is not to be embarked upon lightly.

BIOPHYSICAL TECHNIQUES

A number of physical modalities have been applied to the treatment of vitreous haemorrhage. These include diathermy, cryotherapy, photocoagulation and ultrasound.

Diathermy was first used to treat clinical vitreous haemorrhage in 1954[153]. Forgacs[163] later described an experimental study of rabbit vitreous haemorrhage, in which a faster rate of clot removal was observed in eyes treated with diathermy than in untreated control eyes. The significance of these results is, however, in some doubt, since only three rabbits were studied in each group at two different time periods after treatment (15 days and 120 days), and no account was taken of the considerable interanimal

variation in physiological clearance rates of vitreous clots (see Chapter 7). The necessity for including this variable in studies of vitreous haemorrhage has been emphasised by Jaffe[237]. In spite of this, diathermy has been used widely, particularly by European surgeons[221] but its value is uncertain.

More popular, however, is the use of cryotherapy in the treatment of vitreous haemorrhage[248,430,445,508]. Ricci et al[430] found a beneficial effect in forty-one per cent of cases of vitreous haemorrhages, but Rousselie[445] suggested that it was useful only in cases that were already clearing. Regnault[424] advocated that cryotherapy should be used as a first approach and, if it failed, was to be followed by simple aspiration of the vitreous, and then as a last resort total vitreous removal by vitrectomy.

Baum[39] suggested the use of ultrasound in the treatment of vitreous haemorrhage, since it was found that this form of energy could cause dispersal of vitreous opacities. Difficulties were experienced in concentrating the sound waves on the vitreous opacities without causing damage to surrounding ocular structures. More recent experimental work, however, with focussed transducers has been encouraging[99], and it is likely that clinical trials will soon commence (Coleman, personal communication).

High energy laser beams have also been used to clear vitreous blood. Daily administration of xenon photocoagulation for ten days was reported to hasten the resorption of rabbit vitreous clots, but the study contained insufficient data on the behaviour of control vitreous clots[46].

It is possible that the above physical methods have a potential use in the management of vitreous haemorrhage, since they induce focal tissue damage and will thus elicit an inflammatory response. The

studies described in Chapters 12 and 14 suggest that the low-grade inflammatory response to vitreous blood may be a contributory factor to the delayed resolution of vitreous haemorrhage and thus methods of augmenting this response may enhance reabsorption of the vitreous clot. However, clinical studies have failed to provide good evidence of the value of these methods; it may be that a much greater inflammatory response is required, but this would introduce major risks to the eye itself.

Finally, a report of the value of the hyperosmotic agent, mannitol[57], in the clearance of experimental vitreous haemorrhage has remained unconfirmed. It has been suggested that such agents may promote reabsorption of vitreous clots through an increase in aqueous flow by an as yet undetermined mechanism (ref. 237).

CLOT-LYSING AND ENZYMATIC TECHNIQUES

The use of clot-lysing and enzymatic techniques has been confined mainly to experimental studies. Several substances have been tested, including agents promoting liquefaction of the vitreous gel, agents which cause lysis of the blood clot, and a miscellaneous group of substances directed at other aspects of vitreous haemorrhage absorption (Table A2). The most effective agent within this group was streptokinase-streptodornase[458], but the enzyme was found to cause cataracts and ocular inflammation (uveitis) as had been observed by others using less pure preparations[62, 193]. Streptokinase has been used clinically to clear blood clots from the anterior chamber of the eye (hyphaema) but its use was discontinued due to its high toxicity [154,376]. It was suggested that the marked uveitis which occurred in the human eye after streptokinase injection was the result of the

strongly antigenic nature of this bacterial protein. A similar fibrinolytic agent, urokinase, which is non-antigenic in man was found to be highly effective in the treatment of traumatic hyphaemata and had no significant toxic ocular effects[399,413] (the nature and mode of action of urokinase are described in Chapter 5). More recently, urokinase has been applied to the treatment of persistent vitreous haemorrhage in humans. The initial favourable results[123,152,558] contrasted with similar studies which suggested that urokinase was of little benefit in the treatment of human vitreous clots[91,219]. A later study by Chapman-Smith and Crock[84] reported that urokinase caused subjective and/or objective improvement in vision in nineteen of twenty-seven patients. These authors advocated the use of urokinase as a first line of attack in persistent vitreous haemorrhage, with vitreous surgery reserved for those who fail to respond.

Since the failure of vitreous clots to resolve naturally may be partly due to low levels of fibrinolytic activity within the vitreous (see Chapters 7,8,9), the use of urokinase in this condition would appear to have a rational basis. Although Koziol et al[268] found that urokinase was ineffective in clearing experimental vitreous clots in monkeys, recent experiments in rabbits have shown that a significantly more rapid rate of vitreous clot lysis occurred in eyes treated with intravitreal urokinase than in saline-treated control eyes or in eyes in which uveitis was induced by non-fibrinolytic agents[148]. The effect of urokinase on rabbit vitreous clots was associated with high fibrinolytic activity and was dose-dependent, suggesting that there was a critical level of fibrinolytic activity, below which clot lysis failed to occur.

The mode of action of urokinase in clearing human vitreous haemorrhage is less clear. Recent editorial comment emphasised that

fibrin has not been detected in human vitreous haemorrhage and thus urokinase is unlikely to act as a fibrinolytic enzyme in this situation [129]. On the contrary, electron microscopical studies have shown that fibrin does occur, at least within recent human vitreous haemorrhages[577](Forrester, unpublished observation). Consequently, a substrate for plasmin-mediated fibrinolysis via urokinase may be present in fresh vitreous clots. Urokinase has, however, proved of value in cases where the vitreous opacity was present for over four years[84,152] and it is difficult to conceive of fibrin remaining unaltered in the vitreous for this length of time. The lack of suitable human pathological material for study must leave this question unanswered.

The role of urokinase in promoting clearance of red cells and in the dissolution of fine collagenous membranes with vitreous clots is also difficult to explain. Plasmin-mediated chemotaxis and the resulting inflammatory response (see Chapter 9) may be important during this phase of resolution, since inflammatory cells are known to secrete haemolysins and collagenase (see Chapter 14). Patients treated with intravitreal urokinase frequently develop a marked uveitic response[84,152] and it is likely that this inflammatory reaction influences the clearance of the vitreous opacity. A determining factor, therefore, in the response to urokinase is the extent to which vitreous membranes develop after clot formation (see Chapters 12,13). The use of ultrasound in the selection of cases for treatment increases the rate of success[84].

Complications which may arise from the use of urokinase in vitreous haemorrhage include a sixty-seven per cent incidence of hypopyon uveitis which is transitory, and a twelve per cent incidence of cataract[84,152]. In their series of thirty-four treated eyes,

Chapman-Smith and Crock observed a worsening of the visual status in three cases.

CONCLUSION

Current methods of treatment now offer a measure of hope to the patient blinded by a non-resolving vitreous haemorrhage. The place of vitreous surgery in the treatment of simple vitreous haemorrhage seems well established, but this must be offset by the considerable risk of surgical complications. The value of urokinase therapy has not been critically tested by controlled clinical trial. Nevertheless, there is increasing evidence of its efficacy when used in open trial conditions, and current opinion supports the view that it can be effective in the treatment of vitreous haemorrhage[130]. For the future, urokinase merits further evaluation in appropriately selected patients under rigorous screening procedures.

TABLE 15.2

CLOT-LYSING AND ENZYMATIC AGENTS USED

IN TREATMENT OF EXPERIMENTAL VITREOUS HAEMORRHAGE

VITREOUS GEL LIQUEFACTION	REFERENCE
Collagenase	62
hyaluronidase	62,193,458,529
trypsin	62,83,351
α-chymotrypsin	388,451
CLOT-LYSING AGENTS	
Streptokinase-streptodornase	62,300,458
fibrinolysin	351
urea	62
MISCELLANEOUS	
Saponin	62
phenyl-hydrazine	62
thyroxine	529
deferoxamine	565

TABLE 15.1

COMPLICATIONS OF VITREOUS SURGERY

INTRAOPERATIVE COMPLICATIONS	REFERENCE
Ciliary body laceration	498
lens damage	302,309
corneal lesion in aphakie eyes	302,309
uncontrollable intraocular haemorrhage	332,395
retinal tears or dialyses	302,309,332,395,498
vitreous incarceration	499
POSTOPERATIVE COMPLICATIONS	
retinal traction from sclerotomy	498
fibrous/fibrovascular ingrowth from sclerotomy	410,499
endophthalmitis	302,309,332,395
phthisis bulbi	302,309,332,395
elevated intraocular pressure	302,309,332,395
rubeosis iridis	302,309,332,395
major intraocular haemorrhage	302,309,332,395
recurrent small haemorrhages	302,309,332,395
persistent corneal oedema	302,309,332,395
cystoid macular oedema and macular pucker	302,309,332,395
hypotony	302,309,332,395

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